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1. A process of preparing unsaturated fatty acids, which comprises introducing, into an organism, at least one isolated nucleic acid sequence encoding a polypeptide having $\Delta 6$ -desaturase activity, selected from the group consisting of:
 - a) a nucleic acid sequence having the sequence shown in SEQ ID NO: 1,
 - b) nucleic acid sequences which, as a result of the degeneracy of the genetic code, are derived from the [lacuna] in SEQ ID NO: 1,
 - c) derivatives of the nucleic acid sequence shown in SEQ ID NO: 1 which encode polypeptides with the amino acid sequences shown in SEQ ID NO: 2 and have at least 50% homology at the amino acid level without substantially reducing the enzymatic action of the polypeptides,and culturing this organism, where the cultured organism contains at least 1 mol% of unsaturated fatty acids based on the total fatty acid content in the organism.
2. The process as claimed in claim 1, wherein the nucleic acid sequence is derived from a plant or algae.
3. The process as claimed in claim 1, wherein the nucleic acid sequence is derived from *Physcomitrella patens*.
4. The process as claimed in claim 1, wherein the organism is an organism selected from the group consisting of bacterium, fungus, ciliate, algae, cyanobacterium, animal or plant.
5. The process as claimed in claim 1, wherein the organism is a plant or algae.

6. The process as claimed in claim 1, wherein the organism is an oil crops [sic].
7. The process as claimed in claim 1, wherein the cultured organism contains at least 5% by weight of unsaturated fatty acids based on the total fatty acid content in the organism.
8. The process as claimed in claim 1, wherein the unsaturated fatty acids are isolated from the organism.
9. A transgenic organism selected from the group consisting of plants, fungi, ciliates, algae, bacteria, cyanobacteria or animals comprising at least one isolated nucleic acid sequence encoding a polypeptide with $\Delta 6$ -desaturase activity, selected from the group consisting of:
 - a) a nucleic acid sequence having the sequence shown in SEQ ID NO: 1,
 - b) nucleic acid sequences which, as a result of the degeneracy of the genetic code, are derived from the [lacuna] in SEQ ID NO: 1,
 - c) derivatives of the nucleic acid sequence shown in SEQ ID NO: 1 which encode polypeptides with the amino acid sequences shown in SEQ ID NO: 2 and have at least 50% homology at the amino acid level without substantially reducing the enzymatic action of the polypeptides.
10. A transgenic organism as claimed in claim 9, wherein the organism is a plant or algae.
11. An oil, lipid or fatty acid or a fraction thereof, prepared by the process as claimed in claim 1.
12. The use of the oil, lipid or fatty acid composition as claimed in claim 11 or of a transgenic organism in feed, foodstuffs, cosmetics or pharmaceuticals.

PATENT COOPERATION TREATY

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

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Date of mailing (day/month/year) 14 February 2001 (14.02.01)	
International application No. PCT/EP00/06223	Applicant's or agent's file reference 0050/050461
International filing date (day/month/year) 04 July 2000 (04.07.00)	Priority date (day/month/year) 06 July 1999 (06.07.99)
Applicant HEINZ, Ernst et al	

1. The designated Office is hereby notified of its election made:

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11 December 2000 (11.12.00)

☐ in a notice effecting later election filed with the International Bureau on:

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☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

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PATENT COOPERATION TREATY

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Administrative Instructions, Section 422)

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To:

BASF PLANT SCIENCE GMBH
67056 Ludwigshafen
ALLEMAGNE

Date of mailing (day/month/year) 20 August 2001 (20.08.01)	IMPORTANT NOTIFICATION
Applicant's or agent's file reference 0050/050461	
International application No. PCT/EP00/06223	International filing date (day/month/year) 04 July 2000 (04.07.00)

1. The following indications appeared on record concerning:

☒ the applicant ☐ the inventor ☐ the agent ☒ the common representative

Name and Address BASF AKTIENGESELLSCHAFT D-67056 Ludwigshafen Germany	State of Nationality DE	State of Residence DE
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

☒ the person ☐ the name ☐ the address ☐ the nationality ☐ the residence

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(21) International Application Number: PCT/GB98/03507 (22) International Filing Date: 24 November 1998 (24.11.98) (30) Priority Data: 9724783.7 24 November 1997 (24.11.97) GB (71) Applicant (for all designated States except US): UNIVERSITY OF BRISTOL [GB/GB]; Senate House, Tyndall Avenue, Bristol BS8 1TH (GB). (72) Inventor; and (75) Inventor/Applicant (for US only): NAPIER, Johnathan, A. [GB/GB]; I ACR-Long Ashton Research Station, Dept. of Agriculture Sciences, University of Bristol, Long Ashton, Bristol BS41 9AF (GB). (74) Agents: DEAN, John, Paul et al.; Withers & Rogers, 4 Dyer's Buildings, Holborn, London EC1N 2QP (GB).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
(54) Title: DESATURASE GENES AND THEIR USE (57) Abstract cDNA encoding <i>C. elegans</i> Δ^6 desaturase has been cloned and sequenced, and the Δ^6 desaturase amino acid sequence has been determined. The <i>C. elegans</i> Δ^6 desaturase has a surprisingly low level of sequence identity with the known borage Δ^6 desaturase. The <i>C. elegans</i> Δ^6 desaturase has been expressed in yeast. It and other desaturases can be cloned in host organisms (e.g. plants) and can be used to provide useful metabolites.		

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DESATURASE GENES AND THEIR USE

The present invention relates, *inter alia*, to novel desaturases and to uses thereof.

Over the last few years a number of microsomal and soluble fatty acid desaturases have been isolated from higher plants, most notably from *Arabidopsis thaliana*. This has resulted from a combined genetic and biochemical approach to the generation and complementation of mutant *Arabidopsis* lines defective in fatty acid desaturation or elongation (Somerville C, Browse J (1996) *Trends Cell Biol.* 6, 148-1153). The importance of this approach has been validated by the isolation and characterisation of genes encoding microsomal desaturases such the Δ^{12} (Okuley J, *et al*, (1994), *Plant Cell* 6, 147-158) and Δ^{15} (Arondel V, *et al*, (1992) *Science* 258, 1353-1355) desaturases (encoded by the FAD2 and FAD3 genes respectively), enzymes which had previously proved intractable to classical purification techniques on account of their hydrophobicity. The isolation of these and related enzymes, such as the Δ^{12} hydroxylase from *Ricinus communis* (van de Loo FN *et al* (1995) *Proc. Natl. Acad. Sci USA* 92, 6743-6747), has allowed the identification of a number of conserved motifs in plant microsomal desaturases, most notably the so-called "histidine boxes" (Shanklin, J *et al* (1997) *Proc Natl. Acad. Sci USA.* 92, 6743-6747). Proteins containing these motifs can be classified as di-iron centre-containing enzymes (Shanklin, J *et al* (1997) *Proc. Natl, Acad Sci. USA* 94, 2981-1986).

WO93/11245 (Du Pont) discloses various nucleic acid fragments encoding desaturases, particularly Δ^{12} and Δ^{15} desaturases, which have been isolated from various plants. Recently a cDNA clone was isolated from the plant borage, (*Borago officinalis*) which accumulates γ -linoleic acid (GLA), using highly degenerate PCR against these histidine motifs. US5614393 (Rhone-Poulenc Agrochimie) discloses and claims the nucleotide sequence of borage Δ^6 desaturase. Whilst the specification suggests that Δ^6 desaturase-encoding nucleic acids might be isolated from animal cells without difficulty by the skilled person no suitable animal cells are suggested (in contrast to suggested fungal

and bacterial cells) and there is no disclosure of the isolation of such nucleic acids from animal cells. The isolated DNA clone was shown by heterologous expression in transgenic tobacco to encode a microsomal Δ^6 desaturase (Sayanova O *et al* (1997) *Proc. Natl. Acad. Sci. USA.* **94**, 4211-4216). Desaturation at the Δ^6 position is an unusual modification in higher plants, occurring only in a small number of species such as borage, evening primrose (*Oenothera spp.*) and redcurrant (*Ribes spp.*), which accumulate the Δ^6 -unsaturated fatty acids GLA and octadecatetraenoic acid (OTA:18:^{4,6,9,12,15}, also known as stearidonic acid) in the seeds and/or leaves.

GLA is a high value plant fatty acid, and is widely used in the treatment of a number of medical conditions, including eczema and mastalgia. It has been postulated that the application of GLA replaces the loss of, or meets an increased requirement for, endogenous Δ^6 -unsaturated fatty acids (Horrobin, D.F. (1990) *Rev. Contemp. Pharmacother.* **1**: 1-45).

For reference purposes Figure 5 is provided to show in simplified form a metabolic pathway believed to occur in certain organisms (including humans) and involving Δ^6 desaturases. It can be seen that GLA can be synthesised *in vivo* from linoleic acid under the action of a Δ^6 desaturase and that GLA can be used to synthesise dihomog-LA, which can be converted to arachidonic acid under the influence of a Δ^5 desaturase. Arachidonic acid is a precursor of various important eicosanoids (including prostaglandins and leucotrienes). Δ^6 desaturase also converts α linoleic acid into OTA. Thus it is clear that the Δ^6 desaturase is the first committed step on the biosynthetic pathway of these biologically active molecules (see Fig. 5).

The sequence of the previously isolated borage microsomal Δ^6 desaturase differs from previously characterised plant microsomal desaturases/hydroxylases in that it contains an N-terminal extension which shows homology to cytochrome b₅, and also in that the third (most C-terminal) histidine box varies from the consensus (Shanklin J *et al* (1997) *Proc. Natl. Acad. Sci. USA* **94**, 2981-1986) H-X-X-H-H, with a glutamine replacing the first histidine. This was also observed in the case of the cyanobacteria *Synechocystis* Δ^6 desaturase (GenBank ID; L11421). WO93/06712 (Rhone Poulenc Agrochimie) discloses

an isolated nucleic acid encoding a Δ^6 desaturase isolated from the *Synechocystis*, and claims bacterial Δ^6 desaturases and their uses.

Although Δ^6 fatty acid desaturation is an unusual modification in higher plants, it is believed to be common in animals. The essential fatty acid linoleic acid ($18:2 \Delta^{9,12}$) is desaturated to GLA by a Δ^6 desaturase as a first step in the biosynthetic pathway of the eicosanoids (which include prostagladins and leucotrienes). This results in the rapid metabolism of GLA (to di-homo-GLA and arachidonic acid; i.e. $20:3\Delta^{8,11,14}$ and $20:4 \Delta^{5,8,11,14}$ respectively). Accumulation of GLA is therefore not usually observed.

The nematode worm *Caenorhabditis elegans* is extremely useful in that it has well understood genetics and has many similarities with higher animals such as humans and is therefore extremely useful in the development of desaturases for use in such animals.

According to the present invention, there is provided a polypeptide having desaturase activity, which comprises the amino acid sequence shown in Figure 1.

The amino acid sequence shown in Figure 1 is that of a Δ^6 desaturase that is present in the nematode worm *Caenorhabditis elegans*. This is highly significant since prior to the present invention no successful sequencing or purification of an animal Δ^6 desaturase had been reported. As *C. elegans* does not accumulate GLA isolation of a Δ^6 desaturase from it was an unexpected target for isolating desaturases gene in.

The desaturase of the invention is significantly different from known desaturases. The homology between the Δ^6 desaturase of the invention and the microsomal Δ^{12} and Δ^{15} desaturases from *Arabidopsis* described in WO93/11245 are 24% and 16% respectively as determined using the BESTFIT program. The Δ^6 desaturase gene of the present invention shows 21% identity with the *C.elegans* FAT-1 desaturase described in Spsychalla, J. P. *et al* Proc. Natl Acad. Sci **94** 1142-1147 paper. The sequence homology between the Δ^6 desaturase of the present invention and the *Synechochocystis* Δ^6 described in WO93/06712 is only 23%.

According to another aspect of the invention there is provided therefore an isolated animal Δ^6 desaturase.

The amino acid sequence shown in Figure 1 is also of significance because it has a very low level of sequence identity with the borage Δ^6 desaturase (the only other eukaryotic Δ^6 desaturase to have been sequenced prior to the present invention). Indeed, this level of sequence identity is below 32 %. At such a low level of identity it might be expected that the two polypeptides would have completely different functions. Unexpectedly, both have Δ^6 desaturase activity.

The present invention is, however, not limited to a Δ^6 desaturase having the sequence shown in Figure 1. It also includes other desaturases having at least 32% sequence identity therewith. Preferred polypeptides of the present invention have at least 40 % or more preferably at least 50% amino acid sequence identity therewith. More preferably the degree of sequence identity is at least 75%. Sequence identities of at least 90%, at least 95% or at least 99% are most preferred.

For the purposes of the present invention, sequence identity (whether amino acid or nucleotide) can be determined by using the "BESTFIT" program of the Wisconsin Sequence Analysis Package GCG 8.0.

Where high degrees of sequence identity are present there may be relatively few differences in amino acid sequence. Thus for example there may be less than 20, less than 10, or even less than 5 differences.

Fragments of the polypeptides described above are also within the scope of the present invention, provided that they have desaturase activity, that is to say they have the ability to introduce a double bond into a substrate at a specific position as determined by GCMS. What is the lowest limit for activity. These fragments are preferably at least 100 amino acids long. More preferably, the fragments are at least 150 amino acids long.

In summary, a polypeptide of the present invention has desaturase activity and:

- a) comprises the amino acid sequence shown in Figure 1;

- b) has one or more amino acid deletions, insertions or substitutions relative to a polypeptide as defined in a) above, but has at least 32% amino acid sequence identity therewith; or
- c) is a fragment of a polypeptide as defined in a) or b) above, which is at least 100 amino acids long.

The term "polypeptide" is used herein in a broad sense to indicate that a particular molecule comprises a plurality of amino acids joined together by peptide bonds. It therefore includes within its scope substances, which may sometimes be referred to in the literature as peptides, polypeptides or proteins.

Desirably a polypeptide of the present invention will have a cytochrome domain. A cytochrome domain can be defined as an electron-transporting domain that contains a heme prosthetic group. Preferably a cytochrome b domain is present. More preferably a cytochrome b₅ domain is present (desirably this includes a H-P-G-G-X₁₅-F-X₃₋₆-H, where X is any amino acid, motif). A cytochrome b₅ domain is present in both the borage Δ^6 desaturase and in the *C. elegans* Δ^6 desaturase amino acid sequence shown in Figure 2B. The cytochrome b₅ domain is preferably an N-terminal domain – i.e. it is closer to the N-terminal end of the desaturase than to the C-terminal end. This contrasts with other desaturases. For example, yeast Δ^9 desaturase, has a C-terminal cytochrome b₅ domain and plant Δ^{12} and Δ^{15} desaturases which do not have any b₅ domain.

A polypeptide of the present invention preferably has one or more (most preferably three) histidine boxes. One of these may have an H→Q substitution. (This provides a variant histidine box that is believed to be conserved over a range of animal / plant species.)

Polypeptides of the present invention can have any regiospecificity including *cis/trans* activity although it is preferred that they are front end desaturases that introduce a double bond between the C3 and C7 positions, measured from the COOH (Δ end) of the group. A skilled person is readily able to distinguish between different desaturases by determining the different positions of double bonds introduced by the desaturases. This can be done by known analytical techniques e.g. by using gas chromatography and mass spectrometry.

Particularly preferred desaturases of the invention are Δ^6 desaturases.

Desirably the desaturases occur naturally in one or more organisms that do not accumulate GLA (i.e. where GLA may be produced, but is not normally detectable because it is very quickly metabolised). Such desaturases may occur naturally in one or more animals. The desaturases occur naturally in one or more nematodes, e.g. in *C. elegans*.

In order to appreciate the scope of the present invention more fully, polypeptides within the scope of each of a), b) and c) above will now be discussed in greater detail.

Polypeptides within the scope of a)

A polypeptide within the scope of a) may consist of the amino acid sequence shown in Figure 1 or may have an additional N-terminal and/or an additional C-terminal amino acid sequence.

Additional N-terminal or C-terminal sequences may be provided for various reasons and techniques for providing such additional sequences are well known in the art. Such techniques include using gene-cloning techniques whereby nucleic acid molecules are ligated together and are then used to express a polypeptide in an appropriate host.

Additional sequences may be provided in order to alter the characteristics of a particular polypeptide. This can be useful in improving expression or regulation of expression in particular expression systems. For example, an additional sequence may provide some protection against proteolytic cleavage.

Additional sequences can also be useful in altering the properties of a polypeptide to aid in identification or purification. For example, a signal sequence may be present to direct the transport of the polypeptide to a particular location within a cell or to export the polypeptide from the cell. Different signal sequences can be used for different expression systems.

Another example of the provision of an additional sequence is where a polypeptide is linked to a moiety capable of being isolated by affinity chromatography. The moiety may be an epitope and the affinity column may comprise immobilised antibodies or immobilised antibody fragments that bind to said epitope (desirably with a high degree of specificity). The

resultant fusion protein can usually be eluted from the column by addition of an appropriate buffer.

Additional N-terminal or C-terminal sequences may, however, be present simply as a result of a particular technique used to obtain a polypeptide of the present invention and need not provide any particular advantageous characteristic.

Polypeptides within the scope of b)

Turning now to the polypeptides defined in b) above, it will be appreciated that these are variants of the polypeptides given in a) above.

Various changes can often be made to the amino acid sequence of a polypeptide which has a desired property in order to produce variants which still have that property. Such variants of the polypeptides described in a) above are within the scope of the present invention and are discussed in greater detail in sections (i) to (iii) below. They include allelic and non-allelic variants.

(i) Substitutions

An example of a variant of the present invention is a polypeptide as defined in a) above, apart from the substitution of one or more amino acids with one or more other amino acids.

The skilled person is aware that various amino acids have similar characteristics. One or more such amino acids of a polypeptide can often be substituted by one or more other such amino acids without eliminating a desired property of that polypeptide (such as desaturase activity).

For example, the amino acids glycine, alanine, valine, leucine and isoleucine can often be substituted for one another (amino acids having aliphatic side chains). Of these possible substitutions it is preferred that glycine and alanine are used to substitute for one another (since they have relatively short side chains) and that valine, leucine and isoleucine are used to substitute for one another (since they have larger aliphatic side chains which are hydrophobic). Other amino acids that can often be substituted for one another include phenylalanine, tyrosine and tryptophan (amino acids having aromatic side chains); lysine, arginine and histidine (amino acids having basic side chains); aspartate and glutamate (amino

acids having acidic side chains); asparagine and glutamine (amino acids having amide side chains); and cysteine and methionine (amino acids having sulphur containing side chains).

Substitutions of this nature are often referred to as "conservative" or "semi-conservative" amino acid substitutions.

(ii) Deletions

Amino acid deletions can be advantageous since the overall length and the molecular weight of a polypeptide can be reduced whilst still retaining a desired activity. This can enable the amount of polypeptide required for a particular purpose to be reduced.

(iii) Insertions

Amino acid insertions relative to a polypeptide as defined in a) above can also be made. This may be done to alter the nature of the polypeptide (e.g. to assist in identification, purification or expression).

Polypeptides incorporating amino acid changes (whether substitutions, deletions or insertions) relative to the sequence of a polypeptide as defined in a) above can be provided using any suitable techniques. For example, a nucleic acid sequence incorporating a desired sequence change can be provided by site-directed mutagenesis. This can then be used to allow the expression of a polypeptide having a corresponding change in its amino acid sequence.

Polypeptides within the scope of c)

As discussed *supra*, it is often advantageous to reduce the length of a polypeptide. Feature c) of the present invention therefore covers fragments of the polypeptides a) or b) above which are at least 100 amino acids long, but which do not need to be as long as the full length polypeptide shown in Figure 1. Desirably these fragments are at least 200, at least 300 or at least 400 amino acids long.

Various uses of the polypeptides of the present invention will now be described by way of example only.

Polypeptides of the present invention may be used, *inter alia*, in obtaining useful molecules. For example Δ^6 desaturases can be used in obtaining γ -linolenic acid (GLA) or in obtaining metabolites in respect of which GLA is a precursor. For example, octadecatetraenoic acid (OTA; 18:4 $\Delta^{6,9,12,15}$), a member of the *n*-3 (or ω -3) fatty acids may be produced by the Δ^6 -desaturation of α -linolenic acid.

GLA, OTA and their metabolites are useful in medicine. They can be used in the preparation of a medicament for treating a disorder involving a deficiency in GLA or of a metabolite derived *in vivo* from GLA (e.g. an eicosanoid). Disorders which may be treated include eczema, mastalgia, hypercholesterolemia, atherosclerosis, coronary disease, diabetic neuropathy, viral infections, acne, hypertension, cirrhosis and cancer.

The metabolites may be produced *in vivo* in suitable hosts or *in vitro*.

When a metabolite is to be produced *in vitro*, a desaturase of the present invention and its substrate will normally be provided separately and then combined when it is desired to produce the metabolite. The present invention therefore includes within its scope a method of making GLA or OTA comprising using a Δ^6 desaturase of the present invention to convert linoleic acid substrate or α -linolenic acid substrate to GLA or OTA respectively.

When a metabolite is to be produced *in vivo* in a organism such as a plant or animal, the substrate for a desaturase of the present invention will normally be provided by the relevant non-human organism itself. *In vivo* production of the metabolite can therefore be achieved by inserting a gene encoding a desaturase of the present invention into the organism and allowing the organism to express the desaturase. The desaturase can then act on its substrate. It will therefore be appreciated that polypeptides of the present invention can be used to provide desaturase activity in organisms that would normally not possess such activity or to increase the level of desaturase activity in organisms already having some desaturase activity. If desired, a useful metabolite may be purified from such an organism. Alternatively the organism itself may be used directly as a source of the metabolite. Particular cloning techniques that can be used to provide transgenic organisms with desaturase activity are discussed later on.

Polypeptides of the present invention can also be used as indicators of the transformation of an organism. For example, if an organism intended to be transformed does not have a particular desaturase and a nucleic acid intended for use in transformation encodes that desaturase, an assay can be performed after attempted transformation to determine whether or not the desaturase is present. Thus, in the case of the Δ^6 desaturase, an assay for the presence of GLA may be performed and GLA can serve as a simple marker for the presence of a functional transgene cassette comprising a Δ^6 desaturase encoding sequence.

A further use of the present invention is in providing antibodies. The present invention includes within its scope antibodies that bind to polypeptides of the present invention.

Preferred antibodies bind specifically to polypeptides of the present invention and can therefore be used to purify such polypeptides. (For example, they may be immobilised and used to bind to polypeptides of the present invention. The polypeptides may then be eluted by washing with a suitable eluent under appropriate conditions.)

An antibody or a derivative thereof within the scope of the present invention may be used in diagnosis. For example binding assays using such an antibody or a derivative can be used to determine whether or not a particular desaturase is present. This is useful in diagnosing disorders that arise due to the absence of the functional desaturase.

Antibodies within the scope of the present invention may be monoclonal or polyclonal.

Polyclonal antibodies can be raised by stimulating their production in a suitable animal host (e.g. a mouse, rat, guinea pig, rabbit, sheep, goat or monkey) when a polypeptide of the present invention is injected into the animal. If necessary an adjuvant may be administered together with a polypeptide of the present invention. The antibodies can then be purified by virtue of their binding to a polypeptide of the present invention.

Monoclonal antibodies can be produced from hybridomas. These can be formed by fusing myeloma cells and spleen cells which produce the desired antibody in order to form an immortal cell line. Thus the well-known Kohler & Milstein technique (*Nature* 256 52-55 (1975)) or variations upon this technique can be used.

Techniques for producing monoclonal and polyclonal antibodies that bind to a particular polypeptide are now well developed in the art. They are discussed in standard immunology textbooks, for example in Roitt *et al*, *Immunology* second edition (1989), Churchill Livingstone, London.

In addition to whole antibodies, the present invention includes derivatives thereof which are capable of binding to polypeptides of the present invention. Thus the present invention includes antibody fragments and synthetic constructs. Examples of antibody fragments and synthetic constructs are given by Dougall *et al* in *Tibtech* 12 372-379 (September 1994).

Antibody fragments include, for example, Fab, F(ab')₂ and Fv fragments. (These are discussed, for example, in Roitt *et al* (*supra*)). Fv fragments can be modified to produce a synthetic construct known as a single chain Fv (scFv) molecule. This includes a peptide linker covalently joining V_H and V_L regions, which contributes to the stability of the molecule. Other synthetic constructs that can be used include CDR peptides. These are synthetic peptides comprising antigen-binding determinants. Peptide mimetics may also be used. These molecules are usually conformationally restricted organic rings that mimic the structure of a CDR loop and that include antigen-interactive side chains.

Synthetic constructs include chimaeric molecules. Thus, for example, humanised (or primatised) antibodies or derivatives thereof are within the scope of the present invention. An example of a humanised antibody is an antibody having human framework regions, but rodent hypervariable regions.

Synthetic constructs also include molecules comprising an additional moiety which provides the molecule with some desirable property in addition to antigen binding. For example the moiety may be a label (e.g. a fluorescent or radioactive label). Alternatively, it may be a pharmaceutically active agent.

The present invention also includes nucleic acid molecules within its scope.

Such nucleic acid molecules:

- a) code for a polypeptide according to the present invention; or

- b) are complementary to molecules as defined in a) above; or
- c) hybridise to molecules as defined in a) or b) above.

These nucleic acid molecules and their uses are discussed in greater detail below:

The polypeptides of the present invention can be coded for by a large variety of nucleic acid molecules, taking into account the well-known degeneracy of the genetic code. All of these coding nucleic acid molecules are within the scope of the present invention. Preferred coding nucleic acid molecules encode the polypeptide shown in Figure 1. These include nucleic acid molecules comprising the coding sequence shown in Figure 1 and degenerate variants thereof.

The nucleic acid molecules may be used directly. Alternatively they may be inserted into vectors.

Nucleic acids or vectors containing them may be used in cloning. They may be introduced into non-human hosts to enable the expression of polypeptides of the present invention using techniques known to those skilled in the art. Alternatively, cell free expression systems may be used.

Techniques for cloning, expressing and purifying polypeptides are well known to the skilled person. Various such techniques are disclosed in standard text-books, such as in Sambrook *et al* (*Molecular Cloning* 2nd Edition, Cold Spring Harbor Laboratory Press (1989)); in Old & Primrose (*Principles of Gene Manipulation*, 5th Edition, Blackwell Scientific Publications (1994)); and in Stryer (*Biochemistry*, 4th Edition, W H Freeman and Company (1995)).

By using an appropriate expression system the polypeptides can be produced in a desired form. For example, the polypeptides can be produced by micro-organisms such as bacteria or yeast, by cultured insect cells (which may be baculovirus-infected), or by mammalian cells (such as CHO cells).

However preferred hosts are plants or plant propagating material e.g. oil seed rape, sunflower, cereals including maize, tobacco, legumes including peanut and soybean,

safflower, oil palm, coconut and other palms, cotton, sesame, mustard, linseed, castor, borage and evening primrose, or propagating material therefor.

The technology for providing plants or plant propagating material is now well developed. It is briefly discussed in WO 96/21022, for example. Desaturases isolated from animals have successfully been expressed in plants. For example, Spsychalla, J.P. *et al*, (*supra*) describe the expression of a *C. elegans* desaturase in transgenic Arabidopsis. Additionally, EP0550162 (Pioneer Hi-Bred International, Inc) discloses a chimaric gene construct encoding a Δ^9 desaturase isolated from rat, and plants transformed with the construct for the production of fatty acids. The desaturase described in that publication has only 22% identity with the Δ^6 desaturase of the present invention.

Particular techniques that can be used are discussed below. It will of course be appreciated that such techniques are non-limiting.

(i) Vector systems based on Agrobacterium tumefaciens.

These include Ti based systems, such as pGV3850, in which the T-DNA has been disarmed. Desirably a selectable marker is present (e.g. a marker that provides resistance to an antibiotic).

Intermediate vectors (IVs) may also be used. They tend to be small in size and are therefore usually easier to manipulate than large Ti based vectors. IVs are generally vectors resulting from T-DNA having been cloned into *E. coli* derived plasmid vectors, such as pBR322. IVs are often conjugation-deficient and therefore a conjugation-proficient plasmid (such as pRK2013) may be used to mobilise an IV so that it can be transferred to an *Agrobacterium* recipient. *In vivo* homologous recombination can then occur in an *Agrobacterium* to allow an IV to be inserted into a resident, disarmed Ti plasmid in order that a cointegrate can be produced that is capable of replicating autonomously in the *Agrobacterium*.

Another alternative is to use binary Ti vectors. Here a modified T-DNA region carrying foreign DNA can be provided on a small plasmid that replicates in *E. coli* (e.g. pRK252). This plasmid (sometimes called mini-Ti or micro-Ti) can then be transferred conjugatively

via a tri-parental mating into an *A. tumefaciens* that contains a compatible *vir* gene (providing the *vir* function in *trans*).

Binary vectors without Ti sequences may even be used. Here bacterial *mob* and *oriT* functions may be used to promote plasmid transfer. Again, the *vir* function may be provided in *trans*.

The vector systems discussed above can be used to transfer genes into plants by using the protocol of Horsch *et al.* (*Science* **227**, 1229-31 (1985)) or variants thereof. Here small discs can be punched from the leaves of a dicotyledenous plant, they can be surface-sterilised, and can then be placed in a medium including *A. tumefaciens* that contains recombinant T-DNA in which a foreign gene to be transferred is accompanied by a selectable marker (e.g. the *neo* gene). The discs can then be cultured for 2 days and then transferred to a medium for selecting the selectable marker. (This can be done for a *neo* selectable marker by culturing using a medium containing kanamycin). *A. tumefaciens* can be killed by using a carbenicillin containing medium. Shoots will normally develop from a callus after 2-4 weeks. They can then be excised and transplanted to root-inducing medium and, when large enough can be transplanted into soil.

(ii) Vector systems based on Agrobacterium rhizogenes

These include Ri derived plasmids. Ri T-DNA is generally considered not to be deleterious and therefore such plasmids can be considered as equivalent to disarmed Ti plasmids. An IV co-integrate system based on Ri plasmids has been developed.

(iii) Plant protoplast based transformation systems

Suitable techniques are described in "Plant Gene Transfer and Expression Protocols" ed. H. Jones, Human Press Methods in Molecular Biology, **49**, 1995.

Transformation of plants can be facilitated by removing plant cell walls to provide protoplasts. The cell walls can be removed by any suitable means, including mechanical disruption or treatment with cellulolytic and pectinolytic enzymes. Protoplasts can then be separated from other components by centrifugation and techniques such as electroporation can then be used to transform the protoplasts with heterologous DNA. Under appropriate

culture conditions the transformed protoplasts will grow new cell walls and also divide. Shoots and roots can then be induced and plantlets formed.

(iv) Transfection by biolistics

High velocity microprojectiles carrying DNA or RNA can be used to deliver that DNA or RNA into plant cells. This has allowed a wide variety of transgenic plants to be produced and is suitable for both monocotyledenous and dicotyledenous plants. For example gold or tungsten particles coated with DNA or RNA can be used. Suitable devices for propelling the microprojectiles include gunpowder based devices, electric discharge based devices and pneumatic devices.

(v) Virus based systems

DNA plant virus vectors include cauliflower mosaic viruses (which infect a range of dicots.) and geminiviruses (which infect a wide range of dicots. and monocots). RNA plant viruses are in the majority and include Brome Mosaic Virus (which infects a number of *Graminae*, including barley) and Tobacco Mosaic Virus (which infects tobacco plants).

From the foregoing description it will be appreciated that nucleic acid molecules encoding polypeptides of the present invention can be cloned and expressed in a wide variety of organisms.

In addition to nucleic acid molecules coding for polypeptides of the present invention (referred to herein as "coding" nucleic acid molecules), the present invention also includes nucleic acid molecules complementary thereto. Thus, for example, both strands of a double stranded nucleic acid molecule are included within the scope of the present invention (whether or not they are associated with one another). Also included are mRNA molecules and complementary DNA molecules (e.g. cDNA molecules).

Nucleic acid molecules that can hybridise to one or more of the nucleic acid molecules discussed above are also covered by the present invention. Such nucleic acid molecules are referred to herein as "hybridising" nucleic acid molecules.

A hybridising nucleic acid molecule of the present invention may have a high degree of sequence identity along its length with a nucleic acid molecule within the scope of a) or b) above (e.g. at least 50%, at least 75% or at least 90% sequence identity).

As will be appreciated by those skilled in the art, the greater the degree of sequence identity that a given single stranded nucleic acid molecule has with another single stranded nucleic acid molecule, the greater the likelihood that it will hybridise to a single stranded nucleic acid molecule which is complementary to that other single stranded nucleic acid molecule under appropriate conditions.

Desirably hybridising molecules of the present invention are at least 10 nucleotides in length and preferably are at least 25, at least 50, at least 100 or at least 200 nucleotides in length.

Preferred hybridising molecules hybridise under stringent hybridisation conditions. One example of stringent hybridisation conditions is where attempted hybridisation is carried out at a temperature of from about 35°C to about 65°C using a salt solution that is about 0.9 molar. However, the skilled person will be able to vary such parameters as appropriate in order to take into account variables such as probe length, base composition, type of ions present, etc.

Most preferably, hybridising nucleic acid molecules of the present invention hybridise to a DNA molecule having the coding sequence shown in Figure 1 to an RNA equivalent thereof, or to a complementary sequence to either of the aforesaid molecules.

Hybridising nucleic acid molecules can be useful as probes or primers, for example.

Probes can be used to purify and/or to identify nucleic acids. For example they can be used to identify the presence of all or part of a desaturase gene and are therefore useful in diagnosis.

Primers are useful in amplifying nucleic acids or parts thereof, e.g. by PCR techniques.

In addition to being used as probes or primers, hybridising nucleic acid molecules of the present invention can be used as antisense molecules to alter the expression of polypeptides of the present invention by binding to complementary nucleic acid molecules. (Generally this

can be achieved by providing nucleic acid molecules that bind to RNA molecules that would normally be translated, thereby preventing translation due to the formation of duplexes.)

Hybridising molecules may also be provided as ribozymes. Ribozymes can also be used to regulate expression by binding to and cleaving RNA molecules that include particular target sequences recognised by the ribozymes.

From the foregoing discussion it will be appreciated that a large number of nucleic acids are within the scope of the present invention. Unless the context indicates otherwise, nucleic acid molecules of the present invention may therefore have one or more of the following characteristics:

- 1) They may be DNA or RNA (including variants of naturally occurring DNA or RNA structures, which have non-naturally occurring bases and/or non-naturally occurring backbones).
- 2) They may be single or double stranded.
- 3) They may be provided in recombinant form i.e. covalently linked to a heterologous 5' and/or 3' flanking sequence to provide a chimaeric molecule (e.g. a vector) which does not occur in nature.
- 4) They may be provided without 5' and/or 3' flanking sequences that normally occur in nature.
- 5) They may be provided in substantially pure form, e.g. by using probes to isolate cloned molecules having a desired target sequence or by using chemical synthesis techniques. Thus they may be provided in a form which is substantially free from contaminating proteins and/or from other nucleic acids.
- 6) They may be provided with introns (e.g. as a full-length gene) or without introns (e.g. as cDNA).

The present invention will now be described by way of example only, with reference to the accompanying drawings, Figures 1 to 6 wherein:

Fig 1 shows the DNA sequence and the deduced amino acid sequence of the full length *C. elegans* cDNA pCeD6.1. The positions of the N-terminal cytochrome b₅ domain and the variant third histidine box are underlined. The deduced amino acid sequence of this cDNA is identical to that predicted for residues 1-38 and 68-473 of W08D2.4.

Fig 2A shows a comparison of the deduced amino acid sequences of the *C. elegans* cDNA CeD6.1 and the *C. elegans* predicted protein W08D2.4. (MywormD6=CeD6.1; cew08d2=ORF W08D2.4.)

Fig 2B shows a comparison of the deduced amino acid sequences of the borage Δ^6 desaturase (Sayanova O *et al* (1997) *Proc. Natl. Acad. Sci. USA* **94**, 4211-4216) and the *C. elegans* cDNA CeD6.1. (Boofd6=Borage *officinalis* Δ^6 desaturase; ceeld6=CeD6.1.)

Fig 3 shows methyl esters of total lipids of *S. cerevisiae* grown under inducing conditions (linololate and galactose).

Fig 4 shows GC-MS analysis of the novel peak identified in yeast carrying pYCeD6.1.

Fig 5 shows a simplified version of the metabolism of n-6 essential fatty acids in mammals.

Fig 6 shows fatty acid and methyl esters of leaf material from either control transformed Arabidopsis plant (A) or transformed Arabidopsis plant expressing the *C. elegans* Δ^6 desaturase (B).

Example 1 - Isolation of Δ^6 Desaturase Gene and Expression in Yeast

The NCBI EST sequence database was searched for amino acid sequences using a known borage Δ^6 fatty acid desaturase (Sayanova O *et al* (1997) *supra*) and limiting the search to sequences containing a variant histidine box Q-X-X-H-H.

C. elegans ESTs were identified. They were further characterised by searching the *C. elegans* EST project database (Prof. Y. Kohara lab (National Institute of Genetics, Mishima, Japan); DNA Database of Japan) to identify related cosmid clones.

A partial 448 base pair cDNA clone designated as yk436b12 identified by these searches was obtained from the *C. elegans* EST project, and this was used to screen a *C. elegans* cDNA library (mixed stage; also supplied by Prof Kohara) This indicated that the clone yk436b12 was homologous to part of a gene present on cosmid W08D2 (Genbank accession number Z70271), which forms part of chromosome IV. Bases 21-2957 of cosmid W0D2 are predicted by the protein prediction program Genefinder (Wilson R *et al* (1994) *Nature* 368 32-38 to encode an ORF of 473 residues which is interrupted by 5 introns. Wilson, R. *et al* disclose part of the sequence of chromosome III of *C. elegans*. A number of positives were identified and further purified, and full length clones were confirmed by sequencing to encode a transcript likely to have been transcribed from the gene designated W08D2.4, on cosmid W08D2, as determined by database searching of the genes sequenced by the *C. elegans* genome project.

Examination of this predicted polypeptide (designated W08D2.4 by the Sanger Centre Nematode Sequencing Project, Hinxton, UK) revealed that it had a number of characteristics reminiscent of a microsomal fatty acid desaturase, including three histidine boxes. However, the predicted protein sequence indicated the presence of an N-terminal domain similar to cytochrome b_5 , containing the diagnostic H-P-G-G motif found in cytochrome b_5 proteins (Lederer F (1994) *Biochimie*. 76, 674-692). Since the Δ^6 desaturase isolated by us from borage also contained an N-terminal b_5 domain, this indicated that W0D2.4 may encode a Δ^6 desaturase.

Closer examination of the sequence revealed the presence of the variant third histidine box, with an H→Q substitution (again as observed in the borage Δ^6 desaturase). The degree of similarity between W08D2.4 and the borage Δ^6 desaturase is <52% and is therefore low. The figure of <31% obtained for identity is also low.

Since W08D2.4 was encoded by a gene containing many (6) introns, it was necessary to isolate a full length cDNA to verify the sequence predicted by the Genefinder program , and to also allow the expression of the ORF to define the encoded function.

A cDNA library was screened with the EST insert yk436b12 (generously provided by Prof Y. Kohara) and a number of positive plaques were identified. These were further purified to homogeneity, excised, and the largest inserts (of ~1450 bp) from the resulting rescued phagemids were sequenced. This confirmed that the cDNAs isolated by us were indeed homologous to W08D2.4, with the 5' and 3' ends of the cDNA being equivalent to bases 9 and 3079 of the sequence of cosmid W08D2. Since the ATG initiating codon predicted by the Genefinder program to be the start of gene product W08D2.4 was indeed the first methionine in the cDNA clone, we reasoned that we had isolated a *bona fide* full length cDNA. The DNA sequence and deduced amino acid sequence of one representative cDNA clone (termed pCeD6.1; 1463 bp in length) is shown in Fig 1; the deduced amino acid sequence is identical to that predicted for W08D2.4 over the majority of the protein. The positions of the N-terminal cytochrome *b₅* domain and the variant third histidine box are underlined. The deduced amino acid sequence of this cDNA is identical to that predicted for residues 1-38 and 68-473 of W08D2.4.

However, DNA sequences encoding residues 38-67 (Y-S-I.....L-Y-F) predicted for W08D2.4 are not present in the cDNA clone. This means that the deduced amino acid sequence of CeD6.1 is in fact 443 amino acids long, as opposed to that predicted for W08D2.4, which is 473 residues in length. The only other difference between the two amino acid sequences is an M→V substitution at residue 401, resulting from an A→G base change (base 1211). The two sequences are compared in Fig 2A, as is the deduced amino acid sequence of the borage Δ^6 desaturase and that of CeD6.1 (Fig 2B). The extra sequence predicted for W08D2.4 is likely to derived from incorrect prediction of intron-exon borders.

Note the presence of the H-P-G-G cytochrome *b₅* motif in the N-terminus (encoded by bases 96-108) and the H Q substitution in the third histidine box (encoded by bases 1157-1172).

The coding sequence of W08D2.4 was introduced into the yeast expression vector pYES2 by PCR. Oligonucleotides with 5' overhangs were used to introduce *KpnI* and *SacI* sites at the 5' and 3' ends respectively. The fidelity of the construct was checked by *in vitro* transcription and translation using the TnT system (Promega).

Specifically, clone pCeD6.1 was then used as a template for PCR amplification of the entire predicted coding sequence (443 amino acid residues in length), and cloned into the yeast expression vector pYES2 (Invitrogen) to yield pYCeD6. The fidelity of this PCR-generated sequence was checked *in vitro* transcription/translation of the plasmid, using the T7 RNA polymerase promoter present in pYES2.

Using the Promega TnT coupled transcription/translation system, translation products were generated and analysed by SDS-PAGE and autoradiography as per the manufacturer's instructions. This revealed (data not shown) that the plasmid pYCeD6 generated a product of ~55kD, whereas the control (pYES2) failed to yield any protein products, indicating that the construct was correct.

The resulting plasmid was introduced into yeast (*S. cerevisiae*) by the lithium acetate method (Guthrie C, Fink GR (1991) *Meths Enz* **194**) and expression of the transgene was induced by the addition of galactose. The yeast was supplemented by addition of 0.2 mM linoleate (sodium salt) in the presence of 1% tergitol NP-40.

Transformation and selection of yeast able to grow on uracil-deficient medium revealed yeast colonies carrying the recombinant plasmid pYCeD6 by virtue of the URA3 selectable marker carried by pYES2. Expression of pYCeD6 was obtained by inducing the GAL promoter that is present in pYES2. This was carried out after the cells had been grown up overnight with raffinose as a carbon source, and the medium supplemented by the addition of linoleate (18:2) in the presence of low levels of detergent. This later addition was required since the normal substrate for Δ^6 desaturation is 18:2 fatty acids, which do not normally occur in *S. cerevisiae*.

Yeast total fatty acids were analyzed by GC of methyl esters. Confirmation of the presence of GLA was carried out by GC-MS (Sayanova *et al* (1997) *supra*).

In more detail, the cultures were then allowed to continue to grow after induction, with aliquots being removed for analysis by GC. When methyl esters of total fatty acids isolated from yeast carrying the plasmid pYCeD6 and grown in the presence of galactose and linoleate were analyzed by GC, an additional peak was observed (Fig 3). In Fig. 3 Panel A is yeast transformed with control (empty) vector pYES2, panel B is transformed with pYCeD6.1. The common fatty acid-methyl esters were identified as 16:0 (peak 1), 16:1 (peak 2), 18:0 (peak 3), 18:1 (peak 4), 18:2 (peak 5; supplied exogenously). The additional peak (6) in panel B corresponds to 18:3 GLA, and is indicated by an arrowhead. This had the same retention time as an authentic GLA standard, indicating that the transgenic yeast were capable of Δ^6 -desaturating linoleic acid. No such peaks were observed in any of the control samples (transformation with pYES2). The identity of this extra peak was confirmed by GC-MS, which positively identified the compound as GLA (Fig 4). In the Figure 4 experiment, the sample was analyzed for mass spectra as before (Sayanova O *et al* (1997) *Proc. Natl. Acad. Sci. USA* **94**, 4211-4216), and the data used to search a library of profiles. The sample was identified as GLA. A comparison of the mass spectra of the novel peak (A) and authentic GLA (B) is shown; visual and computer-based inspection revealed them to be identical. This confirms that CeD6.1 encodes a *C. elegans* Δ^6 desaturase, and that this cDNA is likely to be transcribed from the gene predicted to encode ORF W08D2.4, though the deduced amino acid sequence of CeD6.1 is 30 residues smaller than that of W08D2.4

Example 2 -Expression of *C.elegans* Δ^6 desaturase in plants

The coding sequence of the *C. elegans* Δ^6 desaturase was subcloned into a plant expression vector pJD330, which comprises a viral 35S promoter, and a Nos terminator. The resulting cassette or promoter/coding sequence/terminator was then subcloned into the plant binary transformation vector pBin 19, and the resulting plasmid was introduced into *Agrobacterium tumefaciens*. This *Agrobacterium* strain was then used to transform *Arabidopsis* by the vacuum-infiltration of inflorescences. Seeds were harvested and plated onto selective media containing kanamycin. Since pBin 19 confers resistance to this antibiotic, only transformed plant material will grow. Resistant lines were identified and self-fertilized to produce homozygous material. Leaf material was analyzed for fatty acid

profiles using the same method as used for the expression of the nematode desaturase in yeast. Fatty acid methyl esters were separated by GC, and novel peaks shown in Figure 6 identified by comparison with known standards and GCMS. Two novel peaks can be seen in (B) which were identified as γ -linolenic acid (peak 1) and octadecatetraenoic acid (peak 2). These are the products of Δ^6 desaturation of the precursor fatty acids linoleic acid and α -linolenic acid, respectively.

The inventors have shown that a *C. elegans* cDNA (CeD6.1) encodes a Δ^6 desaturase, and that this sequence is identical with the predicted ORF W08D2.4, except for a 30 residue insertion present in the N-terminal region of the latter protein. Whether the deduced amino acid sequence predicted for CeD6.1 represents a splicing variant of W08D2.4, or is a result of a mis-prediction of the intron/exon junctions by the Genefinder programme is unclear. However it is clear that CeD6.1 encodes a Δ^6 desaturase.

The ORF encoded by the this *C. elegans* sequence appears to be related to the higher plant Δ^6 fatty acid desaturase previously isolated by us (Sayanova O *et al* (1997) *supra*), in that they both contain N-terminal domains which show homology to cytochrome b_5 . Microsomal fatty acid desaturases have been demonstrated to use free microsomal cytochrome b_5 as their electron donor (Smith MA, *et al* (1990) *Biochem. J.* **272**, 23-29, Smith MA *et al* (1992) *Biochem. J.* **287**, 141-144), and the vast majority of identified sequences for these enzymes appear not to contain this additional cytochrome b_5 domain (Okuley J *et al* (1994) *Plant Cell* **6**, 147-158, Aronel V. *et al* (1992) *Science* **258**, 1353-1355 and Napier, J.A. *et al* (1997) *Biochemical J*, **328**:717-8).

Prior to the present invention only two examples of cytochrome b_5 -domain-containing desaturases were known, one being the borage Δ^6 desaturase, and the other being the yeast microsomal Δ^9 (OLE1) desaturase (Napier JA *et al* (1997) *Biochemical J*, *supra* and Mitchell AG, Martin CE (1995) *J. Biol. Chem* **270**, 29766-29772). OLE1, however, contains a C-terminal cytochrome b_5 domain (Napier JA *et al* (1997) *Biochemical J*, *in press* and Mitchell AG, Martin CE (1995) *J. Biol. Chem.* **270**, 29766-29772). The reason for the cytochrome b_5 may be that the Δ^6 desaturase is a "front-end" desaturase. (A "front-end" desaturation can be defined as the final desaturation reaction on the fatty acid

chain, usually introducing double bonds between a pre-existing bond and the Δ -end of the carboxy group (Mitchell AG, Martin CE (1995) *J. Biol. Chem* **270**, 29766-29772 and Aitzetmuller K, Tseegsuren, N (1994) *J. Plant Physiol.* **143**, 538-543).)

In any event, it is now believed to be the case that both a variant histidine box and an N-terminal cytochrome b_5 domain are conserved in both animals and plants, as evidenced by their presence in both the borage and nematode Δ^6 desaturases.

The invention may therefore allow the identification of other Δ^6 desaturases and also other "front-end" desaturases to be identified by the presence of these motifs.

Claims

1. A polypeptide having desaturase activity, which:
 - a) has the amino acid sequence shown in Figure 1
 - b) has one or more amino acid deletions, insertions or substitutions relative to a polypeptide as defined in a) above, but has at least 32% amino acid sequence identity therewith; or
 - c) is a fragment of a polypeptide as defined in a) or b) above, which is at least 100 amino acids long.
2. A polypeptide according to claim 1, which has a cytochrome domain.
3. A polypeptide according to claim 2, which has a cytochrome b₅ domain.
4. A polypeptide according to any preceding claim, which has at least one histidine box.
5. A polypeptide according to any preceding claim, which has three histidine boxes.
6. A polypeptide according to any preceding claim, which is a front end desaturase.
7. A polypeptide according to any preceding claim, which is a Δ^6 desaturase.
8. A polypeptide according to any preceding claim, which occurs naturally in an organism that does not accumulate GLA.
9. A polypeptide according to any preceding claim, which occurs naturally in a eukaryote.
10. A polypeptide according to any preceding claim, which occurs naturally in an animal.
11. A polypeptide according to any preceding claim, which occurs naturally in a nematode.
12. A polypeptide according to any preceding claim, which occurs naturally in *C. elegans*.

13. A polypeptide according to claim 1, which consists of the amino acid sequence shown in Figure 1 or of a part thereof.
14. A polypeptide comprising a polypeptide according to any preceding claim, when covalently linked to another moiety.
15. The use of a polypeptide according to any of claims 1 to 14 in raising or selecting antibodies.
16. The use of a polypeptide according to any of claims 1 to 14 as a marker for transformation.
17. The use of a polypeptide according to claim 16 as a marker for plant transformation.
18. An antibody or a derivative thereof which binds to a polypeptide according to any of claims 1 to 14.
19. An antibody or a derivative thereof according to claim 18, for use in diagnosis.
20. A method for assessing whether or not an organism has a polypeptide according to any of claims 1 to 14, comprising determining whether or not the organism has a polypeptide that binds to an antibody or a derivative thereof according to claim 18.
21. A method according to claim 20 in which the organism is a human.
22. A method according to claim 20 or 21 preferred *in vitro*.
23. A polypeptide according to any of claims 1 to 14, for use in medicine.
24. The use of a polypeptide according to any of claims 1 to 14 in the preparation of a medicament for treating a disorder involving a deficiency in GLA in a metabolite derived *in vivo* from GLA.
25. The use of a polypeptide according to claim 23 in which the metabolite is an eicosanoid.

26. The use according to claim 23, 24, or 25 wherein the disorder is eczema, mastalgia, hypercholesterolemia, atherosclerosis, coronary disease, diabetic neuropathy, viral infections, acne, cirrhosis, hypertension and cancer.
27. A method of making GLA comprising using a polypeptide according to any one of claims 1 to 14 to convert linoleic acid to GLA
28. A method of making OTA comprising using a polypeptide according to any one of claims 1 to 14 to convert α linoleic acid to OTA.
29. A nucleic acid molecule which:
- a) codes for a polypeptide according to any of claims claim 1 to 14,
 - b) is the complement of a nucleic acid molecule as defined in a) above, or
 - c) hybridises to a nucleic acid molecule as defined in a) or b) above.
30. A vector comprising a nucleic acid molecule according to claim 29.
31. A host comprising a nucleic acid molecule according to claim 27 or a vector according to claim 30.
32. A host according to claim 31, which is a plant or plant propagating material.
33. A host according to claim 31 or claim 32, which is oil seed rape, sunflower, cereals including maize, tobacco, legumes including peanut and soybean, safflower, oil palm, coconut and other palms, cotton, sesame, mustard, linseed, castor, borage and evening primrose; or which is propagating material for any of the aforesaid.
34. A method for obtaining a polypeptide according to any of claims 1 to 14, comprising incubating a host according to any of claims 31 to 33 under conditions causing expression of said polypeptide and then purifying said polypeptide.
35. The use of nucleic acid molecule according to claim 29 as a probe or as a primer.

36. The use of a nucleic acid molecule according to claim 29 or a vector according to claim 30 for preparing an organism that accumulates GLA or a metabolite derived from GLA in that organism.
37. The use of a nucleic acid molecule according to claim 29 or a vector according to claim 30 for preparing an that is chill resistant .
38. A method of producing a host according to any of claims 31 to 33, comprising incorporating a nucleic acid according to claim 29 or a vector according to claim 30 into an organism.

1/9

10 30 50
GCTCACCAAATGGTCGTCGACAAGAATGCCTCCGGGCTTCGAATGAAGGTCGATGGCAA
M V V D K N A S G L R M K V D G K

70 90 110
ATGGCTCTACCTTAGCGAGGAATTGGTGAAGAAACATCCAGGAGGAGCTGTTATTGAACA
W L Y L S E E L V K K H P G G A V I E Q

130 150 170
ATATAGAAATTCGGATGCTACTCATATTTCCACGCTTTCACGAAGGATCTTCTCAGGC
Y R N S D A T H I F H A F H E G S S Q A

190 210 230
TTATAAGCAACTTGACCTTCTGAAAAAGCACGGAGAGCACGATGAATTCCTTGAGAAACA
Y K Q L D L L K K H G E H D E F L E K Q

250 270 290
ATTGGAAAAGAGACTTGACAAAGTTGATATCAATGTATCAGCATATGATGTCAGTGTTGC
L E K R L D K V D I N V S A Y D V S V A

310 330 350
ACAAGAAAAGAAAATGGTTGAATCATTTCGAAAACTACGACAGAAGCTTCATGATGATGG
Q E K K M V E S F E K L R Q K L H D D G

370 390 410
ATTAATGAAAGCAAATGAAACATATTTCTGTTTAAAGCGATTTC AACACTTTCAATTAT
L M K A N E T Y F L F K A I S T L S I M

430 450 470
GGCATTTCGATTTTATCTTCAGTATCTTGATGGTATATTACTTCTGCATGTTTATTAGC
A F A F Y L Q Y L G W Y I T S A C L L A

490 510 530
ACTTG CATGGCAACAATTCGGATGGTTAACACATGAGTTCTGCCATCAACAGCCAACAAA
L A W Q Q F G W L T H E F C H Q Q P T K

FIG. 1

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550 570 590
GAACAGACCTTTGAATGATACTATTTCTTTGTTCTTTGGTAATTTCTTACAAGGATTTTC
N R P L N D T I S L F F G N F L Q G F S

610 630 650
AAGAGATTGGTGAAGGACAAGCATAACACTCATCACGCTGCCACAAATGTAATTGATCA
R D W W K D K H N T H H A A T N V I D H

670 690 710
TGACGGTGATATCGACTTGGCACCCTTTTCGCATTTATTCCAGGAGATTTGTGCAAGTA
D G D I D L A P L F A F I P G D L C K Y

730 750 770
TAAGGCCAGCTTTGAAAAAGCAATTCTCAAGATTGTACCATATCAACATCTCTATTTTCAC
K A S F E K A I L K I V P Y Q H L Y F T

790 810 830
CGCAATGCTTCCAATGCTCCGTTTCTCATGGACTGGTCAGTCAGTTCAATGGGTATTCAA
A M L P M L R F S W T G Q S V Q W V F K

850 870 890
AGaGAATCAAATGGAGTACAAGGTCTATCAAAGAAATGCATTCTGGGAGCAAGCAACAAT
E N Q M E Y K V Y Q R N A F W E Q A T I

910 930 950
TGTTGGACATTGGGCTTGGGTATTCTATCAATTGTTCTTATTACCAACATGGCCACTTCG
V G H W A W V F Y Q L F L L P T W P L R

970 990 1010
GGTTGCTTATTTTCAATTATTTACAAATGGGAGGAGGCCTTTTGATTGCTCACGTAGTCAC
V A Y F I I S Q M G G G L L I A H V V T

FIG. 1 CONT'D

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1030 1050 1070
TTTCAACCATAACTCTGTTGATAAGTATCCAGCCAATTCTCGAATTTTAAACAACCTTCGC
F N H N S V D K Y P A N S R I L N N F A

1090 1110 1130
CGCTCTTCAAATTTTGACCACACGCAACATGACTCCATCTCCATTTCATTGATTGGCTTTG
A L Q I L T T R N M T P S P F I D W L W

1150 1170 1190
GGGTGGACTCAATTATCAGATCGAGCACCCTTGTTCCTCAACAATGCCACGTTGCAATCT
G G L N Y Q I E H H L F P T M P R C N L

1210 1230 1250
GAATGCTTGCGTGAAATATGTGAAAGAATGGTGCAAAGAGAATAATCTTCCTTACCTCGT
N A C V K Y V K E W C K E N N L P Y L V

1270 1290 1310
CGATGACTACTTTGACGGATATGCAATGAATTTGCAACAATTGAAAAATATGGCTGAGCA
D D Y F D G Y A M N L Q Q L K N M A E H

1330 1350 1370
CATTCAAGCTAAAGCTGCCTAAACAATCTGGGTGTTCAAAAAGTTTTTCTTGTTTTTTT
I Q A K A A *

1390 1410 1430
AAATTTAATCTTTGAAATTATTTGTTTTCCGTCATTCTTCCTCCATTCCCTTTTCTGGT

1450
AGAAATAAAACCTTGTTTTTCAA

FIG. 1 CONT'D

FIG. 2A

PRETTYBOX of: des.msfc(*) November 4, 1997 18:33:04.76

Mywormd6 Cew08d2	MVVDKKNASGL MVVDKKNASGL	RMKVDGKWLY RMKVDGKWLY	LSEELVKKHP LSEELVKKHP	GGAVIEQ GGAVIEQ	YSI YSI	PLNKNIE PLNKNIE	TR TR	GIITRGSSN GIITRGSSN	37 60
Mywormd6 Cew08d2	ALDILYFYRN YRN	SDATHIFHAF SDATHIFHAF	HEGSSQAYKQ HEGSSQAYKQ	LDLLKKHGEH LDLLKKHGEH	DEFLEKQLEK DEFLEKQLEK	RLDKVDINVS RLDKVDINVS			90 120
Mywormd6 Cew08d2	AYDVSVQAQEK AYDVSVQAQEK	KMVESFEKLR KMVESFEKLR	QKLHDDGGLMK QKLHDDGGLMK	ANETYFLFKA ANETYFLFKA	ISTLSIMAF ISTLSIMAF	FYLOYLGWYI FYLOYLGWYI			150 180
Mywormd6 Cew08d2	TSACLLALAW TSACLLALAW	QQFGWLTHEF QQFGWLTHEF	CHQQPTKNRP CHQQPTKNRP	LNDTISLFFG LNDTISLFFG	NFLQGGFSRDW NFLQGGFSRDW	WKDKHNTTHHA WKDKHNTTHHA			210 240
Mywormd6 Cew08d2	ATNVIDHDGD ATNVIDHDGD	IDLAPLEAFI IDLAPLEAFI	PGDLCKYKAS PGDLCKYKAS	FEKAILKKIVP FEKAILKKIVP	YQHLYFTAML YQHLYFTAML	PMLRFSWTGQ PMLRFSWTGQ			270 300
Mywormd6 Cew08d2	SVQWVFKENQ SVQWVFKENQ	MEYKVYQRNA MEYKVYQRNA	FWEQATIVGH FWEQATIVGH	WAWVFYQLFL WAWVFYQLFL	LPTWPLRVAY LPTWPLRVAY	FISQMGGL FISQMGGL			330 360
Mywormd6 Cew08d2	LIAHVVTFNH LIAHVVTFNH	NSVDKYPANS NSVDKYPANS	RILNNFAALO RILNNFAALO	ILTRNNTPS ILTRNNTPS	PFIDWLWGGL PFIDWLWGGL	NYQIEHHLFP NYQIEHHLFP			390 420
Mywormd6 Cew08d2	TMPRCNLNAC TMPRCNLNAC	VKYVKEMCKE MKYVKEMCKE	NNLPYLVDDY NNLPYLVDDY	FDGYAMNLOQ FDGYAMNLOQ	LKNMAEHIOA LKNMAEHIOA	KAA*443 KAA*473			

boofd6 ceeld6	MAAQIKKYIT	SDELKNHDKP	GD	LWISIQGK	AYDVS	.D	WVK	DHPGGGSFPLK	SLAQQEV	TDA	59
MVDKNA	SG	LRMKVDGK	WLYLS	SEELVK		KHPGGGAV	.IE	QYRNSDA	THI 46
boofd6 ceeld6	FVAFHPAS	..TWKNLDDKF	..FTGYYLK	..HGEHDEFLEK	DY	..SVSEVS	..INVSAYDVSV	KDYRKL	LVFEF	F	100
	FHAFHEGSSQ	AYKQLD	LLKK	..HGEHDEFLEK	QLEKRLDKVD	..SVSEVS	..INVSAYDVSV	AQEK	KMVESEF	F	106
boofd6 ceeld6	SKMGLYDKKG	HIMFA	..TLC	FIAMLFAMSV	YGVLF	CEGV	LVHLF	SGCLM	GFLW	IQSGWI	157
	EKLRLQKLHDD	GLMKA	NETYF	LFKAISTLSI	MAFAFY	LQYL	GWYIT	TSACLL	ALAW	QOFGWL	166
boofd6 ceeld6	GHDAGHYMVV	SDSR	LNKFMG	IFAA	NCCLSGI	SIGWMK	WNHN	AHHIA	CNLSLE	YD	217
	THEFCHQQPT	KNRPL	NDTIS	LFFGNF	LQGF	SRD	WWKDKHN	THHA	ATNVID	HDGD	IDLAP 225
boofd6 ceeld6	LVVSSKFFGS	LTSHFYEKRL	TFDSLSRFFV	SYQHWT	FYPI	MCAAR	RLNMYV	QSL	IMLLTKR	277	
	...LF	AF	IPGDLCKYKA	SPEKAILKIV	PYOHLYFTAM	LPMLR	FSWTG	QSV	QWVFKEN	279	
boofd6 ceeld6	NVSYRAHE	..LLG	CLVFSI	WYPL	LVSC	LPN	WGE	RIMFVI	ASLS	VT	327
	QMEYKVYQRN	AFWEQ	ATIVG	HWAW	VFYQL	FL	..LPTWPL	RVAYFI	ISQM	GG	336
boofd6 ceeld6	SLNHFS	SSSVY	VGKPKG	NNW	FEK	QTDG	TLD	HGGL	QFQIEH	HLFP	386
	TFNHNS	VVDKY	PANSRI	LN	NF	AAL	QIL	TTRN	MTPSP	FIDWL	396
boofd6 ceeld6	LRKISP	YVIE	LCKKH	NLPYN	YAS	F	SKANEM	T	LR	T	444
	LNACVK	YVKE	WCKEN	NLPYL	VDDY	F	DGYAM	N	LQ	Q	443

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FIG. 2B

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FIG. 3A

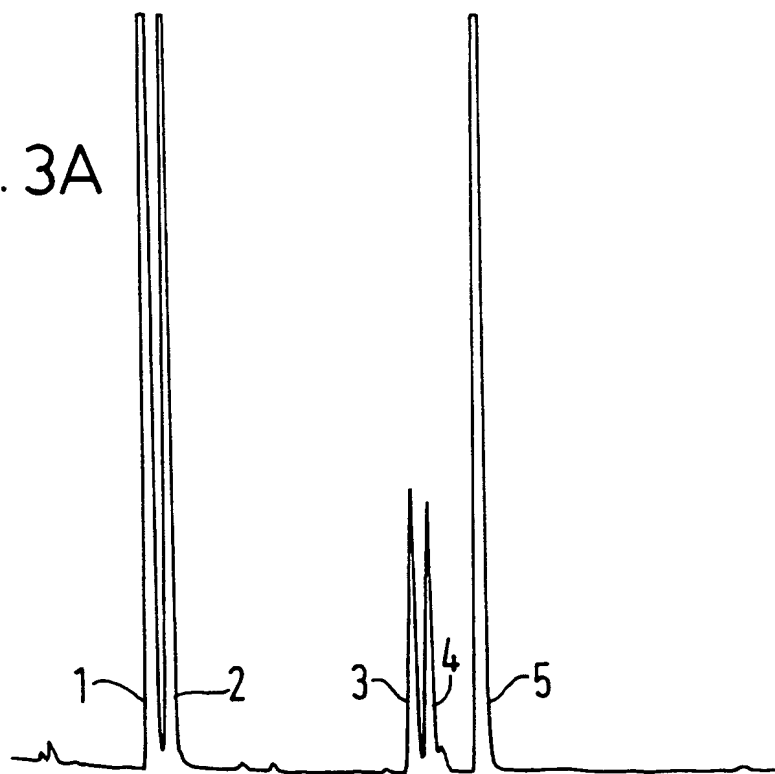
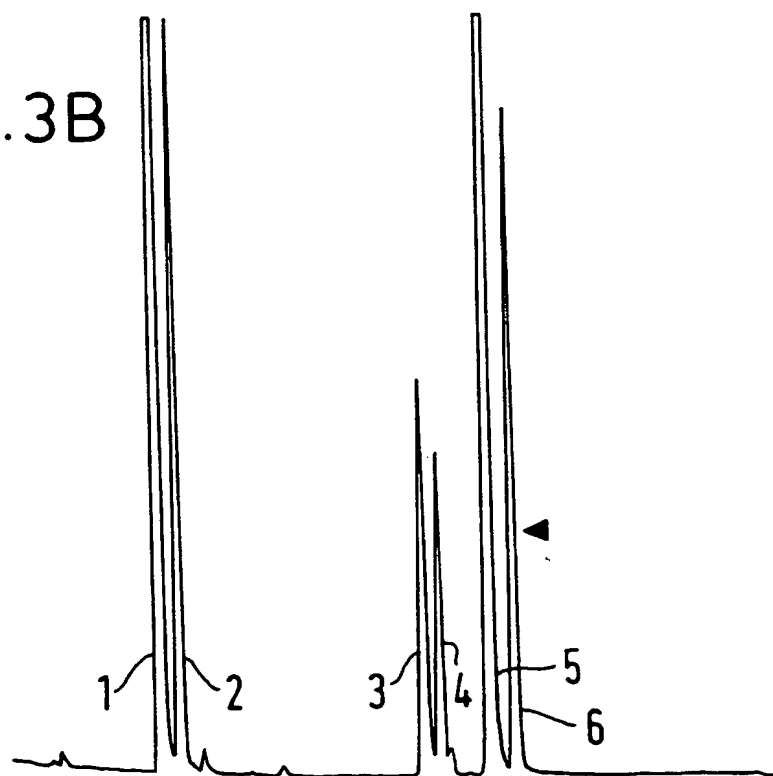


FIG. 3B



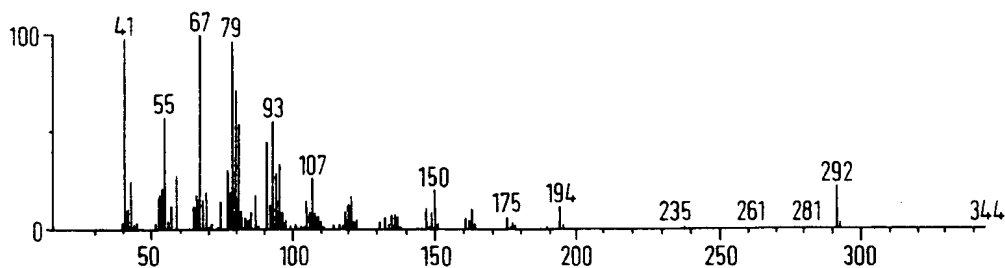
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Run =DOM10004 Scan=738 (Sub) 100%=413600 ADC Mass Range=40-456
 23 Sep 97 3:50 Compacted SLRP +EI 1UL C.E O/N INDUCTION + LA

Serial	Rel. (Sim)	Rel. (Same)	Mol. Wt.	Formula & Name
77275	99	81	292	C19 H32 O2 6, 9, 12-Octadecatrienoic acid, methyl ester
81040	95	74	122	C9 H14 1, 4-Cyclononadiene
43157	95	74	292	C19 H32 O2 6, 9, 12-Octadecatrienoic acid, methyl ester
77274	93	68	292	C19 H32 O2 6, 9, 12-Octadecatrienoic acid, methyl ester
6892	60	35	136	C10 H16 BETA.-FENCHENE
4278	59	33	122	C9 H14 3-Nonen-1-yne, (z)-
25116	55	29	206	C15 H26 5-Pentadecen-7-yne, (z)-
17742	55	29	178	C13 H22 3-Tridecen-1-yne, (z)-
13423	55	29	162	C12 H18 1, 4, 8-Dodecatriene, (E, E, E)-
10169	34	11	150	C11 H18 Cyclopropane, 1-etheny-2-hexenyl-, 1.alpha., 2.beta. (E)
2366	33	10	108	C8 H12 1, 4-Cyclooctadiene, (z, z)-
2372	32	9	108	C8 H12 Bicyclo 5.1.0. oct-3-ene
6909	24	6	136	C10 H16 Cyclooctene, 3-ethenyl-
10171	23	6	150	C11 H18 Cyclohexene, 3-(3-methyl-1-butenyl)-, (E)-
29046	23	6	222	C15 H26 O 5, 10-Pentadecadienal, (z, z)-
17743	21	5	178	C13 H22 3-Tridecen-1-yne, (E)-
10173	20	4	150	C11 H18 Spiro 5.5.undec-1-ene
4281	20	4	122	C9 H14 Bicyclo 5.1.0. octane, 8-methylene-
10192	18	3	150	C11 H18 (-)-2-METHYL-2-BORNENE
4291	18	3	122	C9 H14 1, 2-CYCLONONADIENE
2597	18	3	110	C8 H14 Cyclopentane, (1-methylethylidene)-
7335	12	2	138	C10 H18 4-Decyne
67891	11	2	82	C6 H10 Cyclopropane, 1, 2-dimethyl-3-methylene-, cis-
618	11	2	82	C6 H10 Cyclopropane, 1, 2-dimethyl-3-methylene-, trans-
71289	4	1	136	C10 H16 2-BETA.-PINENE

Run =DOM10004 Scan=738 (Sub) 100%=413600 ADC Mass Range=40-456
 23 Sep 97 3:50 Compacted SLRP +EI 1UL C.E O/N INDUCTION + LA



wileynbs: 77275 Rel(sim):99 Rel(same):81
 6,9,12-Octadecatrienoic acid, methyl ester
 C19 H32 O2 MW=292.240230 CAS=2676417

Me (CH₂)₄ CH CH CH₂ CH CH CH₂ CH CH (CH₂)₄ C(O) OMe

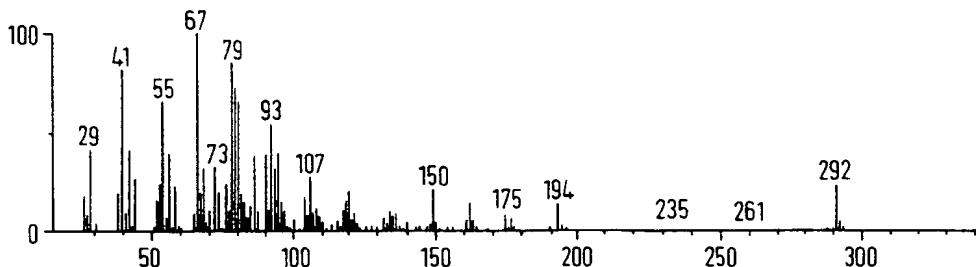


FIG. 4

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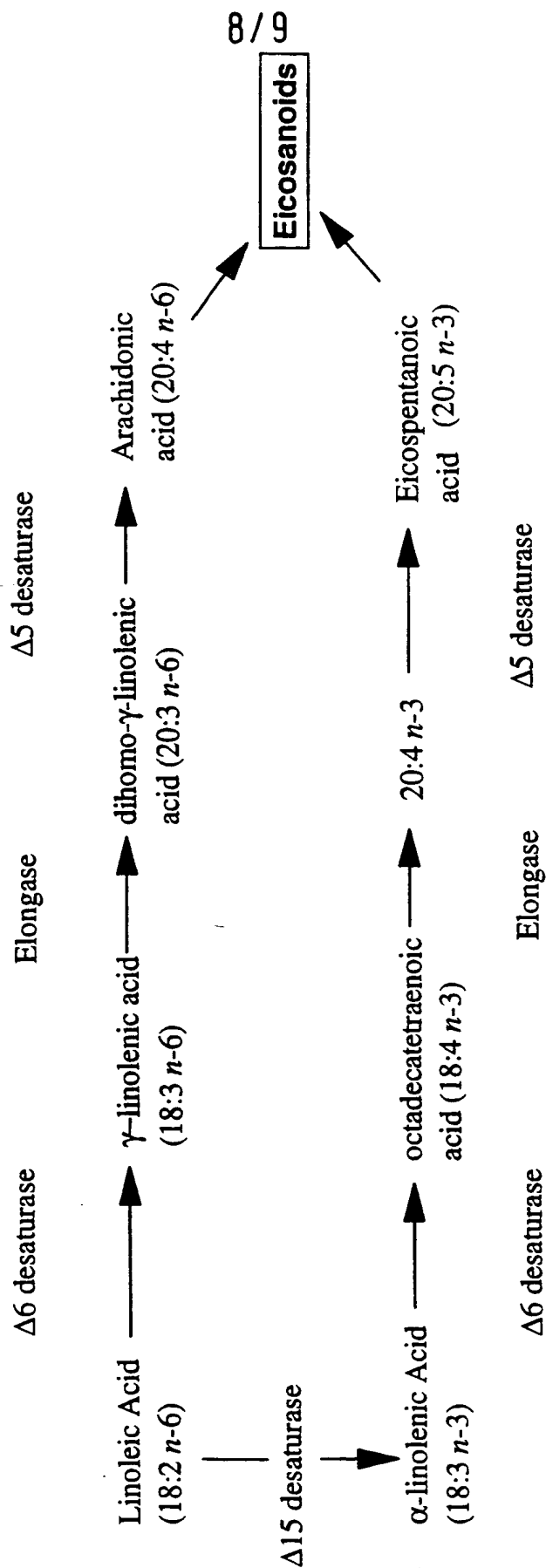


FIG. 5

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FIG. 6A

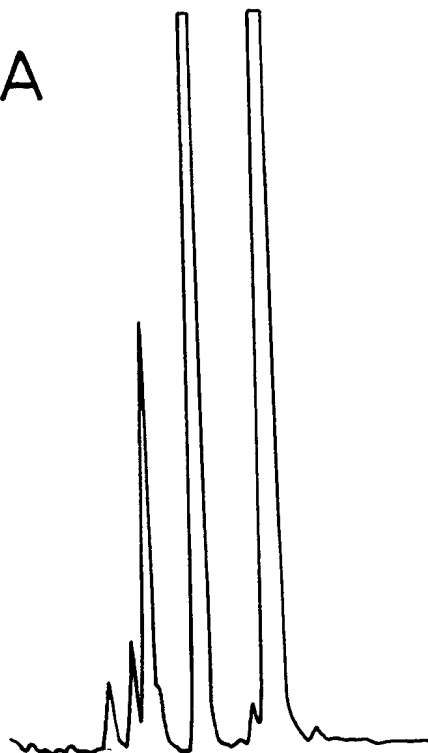
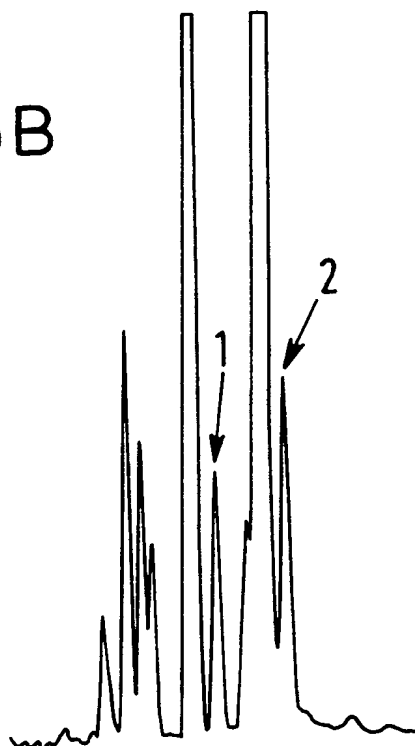


FIG. 6B



INTERNATIONAL SEARCH REPORT

International Application No.

PCT/GB 98/03507

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/53 C12N15/82 C12N9/02 C12Q1/26 G01N33/53
A61K38/44 C07K16/40 C12P7/64 C12N5/10 A01H5/00
C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12Q G01N A61K A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HILLIER, L., ET AL.: "pk42c09.r1 Caenorhabditis briggsae cDNA similar to SP:S35157 S35157 DELTA(6)-DESATURASE - SYNECHOCYSTIS" EMBL ACCESSION NO. R05219, 18 April 1995, XP002099441 see the whole document ---	1-13, 29-31, 38
X	SWINBOURNE, J., ET AL.: "Caenorhabditis elegans cosmid W08D2" EMBL ACCESSION NO. Z70271, 23 March 1996, XP002099442 see translation product for gene W08D2.4 and sequence 1251-1999 --- -/--	1-13, 29-31, 38



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

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PCT/GB 98/03507

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SWINBOURNE, J., ET AL.: "T13F2.1" TREMBL ACCESSION NO. Q94044, 1 February 1997, XP002099443 & EMBL ACCESSION NO. Z81122, 21 October 1996, ---	1-6, 8-13, 29-31
X	KOHARA, Y.: "C. elegans cDNA clone yk436b12 : 5' end, single read" EMBL SEQUENCE ACCESSION NO. C47394, 9 September 1997, XP002099444 see the whole document ---	29-31, 38
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P, X	SAYANOVA, O., ET AL.: "Caenorhabditis elegans delta6-fatty-acid-desaturase mRNA, complete cds." EMBL ACCESSION NO. AF031477, 27 April 1998, XP002099446 see the whole document ---	1-13, 29-31, 38
P, X	-& NAPIER, J.A., ET AL.: "Identification of a Caenorhabditis elegans delta6-fatty-acid-desaturase by heterologous expression in Saccharomyces cerevisiae" BIOCHEMICAL JOURNAL, vol. 330, no. 2, March 1998, pages 611-614, XP002099453 see the whole document ---	1-13, 27, 29-31, 36, 38
P, X	WO 98 46763 A (THURMOND JENNIFER ; CALGENE LLC (US); ABBOTT LAB (US); KNUTZON DEBO) 22 October 1998 see seq IDs 2, 5, and 10. ---	1-9, 13, 23-31, 36, 38
P, X	WO 98 46764 A (THURMOND JENNIFER ; CALGENE LLC (US); ABBOTT LAB (US); KNUTZON DEBO) 22 October 1998 see sequence IDs 2 and 12 ---	1-9, 13, 23-33, 36, 38
	-/--	

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 98/03507

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>SAYANOVA, O., ET AL.: "Expression of a borage desaturase cDNA containing an N-terminal cytochrome b5 domain results in the accumulation of high levels of delta-6-desaturated fatty acids in transgenic tobacco"</p> <p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol. 94, April 1997, pages 4211-4216, XP002099447 WASHINGTON US cited in the application see the whole document</p> <p style="text-align: center;">---</p>	1-38
A	<p>TANAKA, T., ET AL.: "Effects of growth temperature on the fatty acid composition of the free-living nematode <i>Caenorhabditis elegans</i>"</p> <p>LIPIDS, vol. 31, no. 11, - 1996 pages 1173-1178, XP002099448 see page 1176, left-hand column</p> <p style="text-align: center;">---</p>	1-38
A	<p>SPYCHALLA, J. P., ET AL.: "Identification of an animal omega-3 fatty acid desaturase by heterologous gene expression in <i>Arabidopsis</i>"</p> <p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol. 94, February 1997, pages 1142-1147, XP002099449 WASHINGTON US cited in the application see the whole document</p> <p style="text-align: center;">---</p>	1-38
A	<p>EP 0 410 637 A (IRELAND BIORESEARCH ;HEADON DENIS ROBERT (IE); MCSWEENEY FINBARR M) 30 January 1991 see the whole document</p> <p style="text-align: center;">---</p>	15,18-22
A	<p>EP 0 454 102 A (EFAMOL HOLDINGS) 30 October 1991 see the whole document</p> <p style="text-align: center;">---</p>	23-26
A	<p>EP 0 400 547 A (ABBOTT LAB) 5 December 1990 see the whole document</p> <p style="text-align: center;">---</p>	23-26
A	<p>WO 96 21022 A (RHONE POULENC AGROCHIMIE) 11 July 1996 see page 2, line 3 - page 3, line 25 see page 11, line 10 - line 21 see page 12, line 5 - line 23 see page 19, line 25 - page 20, line 10 see page 31 - page 43; figures 4,9</p> <p style="text-align: center;">---</p>	24-38
	-/--	

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PCT/GB 98/03507

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>KODAMA H ET AL: "GENETIC ENHANCEMENT OF COLD TOLERANCE BY EXPRESSION OF A GENE FOR CHLOROPLAST OMEGA-3 FATTY ACID DESATURASE IN TRANSGENIC TOBACCO" PLANT PHYSIOLOGY, vol. 105, 1 January 1994, pages 601-605, XP002001002 see the whole document -----</p>	37

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 98/03507

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
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EP 0454102	A	30-10-1991	AT 87825 T AU 618814 B AU 3597489 A AU 633442 B CA 1334004 A EP 0347056 A ES 2053990 T IE 63303 B JP 2032017 A JP 2796838 B KR 129666 B NZ 239126 A US 4977187 A US 5120760 A	15-04-1993 09-01-1992 14-12-1989 28-01-1993 17-01-1995 20-12-1989 01-08-1994 05-04-1995 01-02-1990 10-09-1998 09-04-1998 27-07-1997 11-12-1990 09-06-1992
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/53, 15/82, A01H 5/00	A2	(11) International Publication Number: WO 96/21022 (43) International Publication Date: 11 July 1996 (11.07.96)
(21) International Application Number: PCT/IB95/01167 (22) International Filing Date: 28 December 1995 (28.12.95) (30) Priority Data: 08/366,779 30 December 1994 (30.12.94) US (71) Applicant: RHONE-POULENC AGROCHIMIE [FR/FR]; 14-20, rue Pierre-Baizet, F-69263 Lyon (FR). (72) Inventors: THOMAS, Terry, L.; 3004 Normand, College Station, TX 77845 (US). REDDY, Avutu, S.; 3902 E. 29th Street #G11, Bryan, TX 77802 (US). NUCCIO, Michael; P.O. Box 553, College Station, TX 77841 (US). NUNBERG, Andrew, N.; 2804 B. Sprucewood Street, Bryan, TX 77801 (US). FREYSSINET, Georges, L.; 21, rue de Nervieux, F-69450 Saint-Cyr-au-Mont-d'Or (FR). (74) Agent: MITSCHERLICH & PARTNER; Sonnenstrasse 33, D-80331 München (DE).		(81) Designated States: AU, BR, CA, CN, JP, RO, RU, UA, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: PRODUCTION OF GAMMA LINOLENIC ACID BY A $\Delta 6$ -DESATURASE (57) Abstract Linoleic acid is converted into γ -linolenic acid by the enzyme $\Delta 6$ -desaturase. The present invention is directed to isolated nucleic acids comprising the $\Delta 6$ -desaturase gene. More particularly, the isolated nucleic acid comprises the promoter, coding region and termination regions of the $\Delta 6$ -desaturase gene. The present invention provides recombinant constructions comprising the $\Delta 6$ -desaturase coding region in functional combination with heterologous regulatory sequences. The nucleic acids and recombinant constructions of the instant invention are useful in the production of GLA in transgenic organisms.		

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CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

1 PRODUCTION OF GAMMA LINOLENIC ACID BY A $\Delta 6$ -DESATURASE

Linoleic acid (18:2) (LA) is transformed into gamma linolenic acid (18:3) (GLA) by the enzyme
5 $\Delta 6$ -desaturase. When this enzyme, or the nucleic acid encoding it, is transferred into LA-producing cells, GLA is produced. The present invention provides nucleic acids comprising the $\Delta 6$ -desaturase gene. More specifically, the nucleic acids comprise the
10 promoters, coding regions and termination regions of the $\Delta 6$ -desaturase genes. The present invention is further directed to recombinant constructions comprising a $\Delta 6$ -desaturase coding region in functional combination with heterologous regulatory sequences.
15 The nucleic acids and recombinant constructions of the instant invention are useful in the production of GLA in transgenic organisms.

Unsaturated fatty acids such as linoleic ($C_{18}\Delta^{9,12}$) and α -linolenic ($C_{18}\Delta^{9,12,15}$) acids are essential
20 dietary constituents that cannot be synthesized by vertebrates since vertebrate cells can introduce double bonds at the Δ^9 position of fatty acids but cannot introduce additional double bonds between the Δ^9 double bond and the methyl-terminus of the fatty
25 acid chain. Because they are precursors of other products, linoleic and α -linolenic acids are essential fatty acids, and are usually obtained from plant sources. Linoleic acid can be converted by mammals into γ -linolenic acid (GLA, $C_{18}\Delta^{6,9,12}$) which can in turn
30 be converted to arachidonic acid (20:4), a critically

1 important fatty acid since it is an essential
precursor of most prostaglandins.

The dietary provision of linoleic acid, by
virtue of its resulting conversion to GLA and
5 arachidonic acid, satisfies the dietary need for GLA
and arachidonic acid. However, a relationship has
been demonstrated between consumption of saturated
fats and health risks such as hypercholesterolemia,
atherosclerosis and other clinical disorders which
10 correlate with susceptibility to coronary disease,
while the consumption of unsaturated fats has been
associated with decreased blood cholesterol
concentration and reduced risk of atherosclerosis.
The therapeutic benefits of dietary GLA may result
15 from GLA being a precursor to arachidonic acid and
thus subsequently contributing to prostaglandin
synthesis. Accordingly, consumption of the more
unsaturated GLA, rather than linoleic acid, has
potential health benefits. However, GLA is not
20 present in virtually any commercially grown crop
plant.

Linoleic acid is converted into GLA by the
enzyme $\Delta 6$ -desaturase. $\Delta 6$ -desaturase, an enzyme of
more than 350 amino acids, has a membrane-bound domain
25 and an active site for desaturation of fatty acids.
When this enzyme is transferred into cells which
endogenously produce linoleic acid but not GLA, GLA is
produced. The present invention, by providing the
gene encoding $\Delta 6$ -desaturase, allows the production of
30 transgenic organisms which contain functional $\Delta 6$ -
desaturase and which produce GLA. In addition to

1 allowing production of large amounts of GLA, the
present invention provides new dietary sources of GLA.

The present invention is directed to
isolated $\Delta 6$ -desaturase genes. Specifically, the
5 isolated genes comprises the $\Delta 6$ -desaturase promoters,
coding regions, and termination regions.

The present invention is further directed to
expression vectors comprising the $\Delta 6$ -desaturase
promoter, coding region and termination region.

10 Yet another aspect of this invention is
directed to expression vectors comprising a $\Delta 6$ -
desaturase coding region in functional combination
with heterologous regulatory regions, i.e. elements
not derived from the $\Delta 6$ -desaturase gene.

15 Cells and organisms comprising the vectors
of the present invention, and progeny of such
organisms, are also provided by the present invention.

A further aspect of the present invention
provides isolated bacterial $\Delta 6$ -desaturase. An
20 isolated plant $\Delta 6$ -desaturase is also provided.

Yet another aspect of this invention
provides a method for producing plants with increased
gamma linolenic acid content.

A method for producing chilling tolerant
25 plants is also provided by the present invention.

Fig. 1 depicts the hydropathy profiles of
the deduced amino acid sequences of Synechocystis $\Delta 6$ -
desaturase (Panel A) and $\Delta 12$ -desaturase (Panel B).
Putative membrane spanning regions are indicated by
30 solid bars. Hydrophobic index was calculated for a

1 window size of 19 amino acid residues [Kyte, et al.
(1982) J. Molec. Biol. 157].

Fig. 2 provides gas liquid chromatography
profiles of wild type (Panel A) and transgenic (Panel
5 B) Anabaena.

Fig. 3 is a diagram of maps of cosmid cSy75,
cSy13 and Csy7 with overlapping regions and subclones.
The origins of subclones of Csy75, Csy75-3.5 and Csy7
are indicated by the dashed diagonal lines.
10 Restriction sites that have been inactivated are in
parentheses.

Fig. 4 provides gas liquid chromatography
profiles of wild type (Panel A) and transgenic (Panel
B) tobacco.

15 Fig. 5A depicts the DNA sequence of a Δ -6
desaturase cDNA isolated from borage.

Fig. 5B depicts the protein sequence of the
open reading frame in the isolated borage Δ -6
desaturase cDNA. Three amino acid motifs
20 characteristic of desaturases are indicated and are,
in order, lipid box, metal box 1, and metal box 2.

Fig. 6 is a dendrogram showing similarity of
the borage Δ 6-desaturase to other membrane-bound
desaturases. The amino acid sequence of the borage
25 Δ 6-desaturase was compared to other known desaturases
using Gene Works (IntelliGenetics). Numerical values
correlate to relative phylogenetic distances between
subgroups compared.

Fig. 7 is a restriction map of 221. Δ 6.NOS
30 and 121. Δ 6.NOS. In 221. Δ 6.NOS, the remaining portion

1 of the plasmid is pBI221 and in 121.Δ6.NOS, the
remaining portion of the plasmid is pBI121.

Fig. 8 provides gas liquid chromatography
profiles of mock transfected (Panel A) and 221.Δ6.NOS
5 transfected (Panel B) carrot cells. The positions of
18:2, 18:3 α, and 18:3 γ(GLA) are indicated.

Fig. 9 provides gas liquid chromatography
profiles of an untransformed tobacco leaf (Panel A)
and a tobacco leaf transformed with 121.Δ6.NOS. The
10 positions of 18:2, 18:3 α, 18:3γ(GLA), and 18:4 are
indicated.

Fig. 10 provides gas liquid chromatography
profiles for untransformed tobacco seeds (Panel A) and
seeds of tobacco transformed with 121.Δ6.NOS. The
15 positions of 18:2, 18:3α and 18:3γ(GLA) are indicated.

The present invention provides isolated
nucleic acids encoding Δ6-desaturase. To identify a
nucleic acid encoding Δ6-desaturase, DNA is isolated
from an organism which produces GLA. Said organism
20 can be, for example, an animal cell, certain fungi
(e.g. Mortierella), certain bacteria (e.g.
Synechocystis) or certain plants (borage, Oenothera,
currants). The isolation of genomic DNA can be
accomplished by a variety of methods well-known to one
25 of ordinary skill in the art, as exemplified by
Sambrook et al. (1989) in Molecular Cloning: A
Laboratory Manual, Cold Spring Harbor, NY. The
isolated DNA is fragmented by physical methods or
enzymatic digestion and cloned into an appropriate
30 vector, e.g. a bacteriophage or cosmid vector, by any
of a variety of well-known methods which can be found

1 in references such as Sambrook et al. (1989).
Expression vectors containing the DNA of the present
invention are specifically contemplated herein. DNA
encoding $\Delta 6$ -desaturase can be identified by gain of
5 function analysis. The vector containing fragmented
DNA is transferred, for example by infection,
transconjugation, transfection, into a host organism
that produces linoleic acid but not GLA. As used
herein, "transformation" refers generally to the
10 incorporation of foreign DNA into a host cell.
Methods for introducing recombinant DNA into a host
organism are known to one of ordinary skill in the art
and can be found, for example, in Sambrook et al.
(1989). Production of GLA by these organisms (i.e.,
15 gain of function) is assayed, for example by gas
chromatography or other methods known to the
ordinarily skilled artisan. Organisms which are
induced to produce GLA, i.e. have gained function by
the introduction of the vector, are identified as
20 expressing DNA encoding $\Delta 6$ -desaturase, and said DNA is
recovered from the organisms. The recovered DNA can
again be fragmented, cloned with expression vectors,
and functionally assessed by the above procedures to
define with more particularity the DNA encoding $\Delta 6$ -
25 desaturase.

As an example of the present invention,
random DNA is isolated from the cyanobacteria
Synechocystis Pasteur Culture Collection (PCC) 6803,
American Type Culture Collection (ATCC) 27184, cloned
30 into a cosmid vector, and introduced by
transconjugation into the GLA-deficient cyanobacterium

1 Anabaena strain PCC 7120, ATCC 27893. Production of GLA from Anabaena linoleic acid is monitored by gas chromatography and the corresponding DNA fragment is isolated.

5 The isolated DNA is sequenced by methods well-known to one of ordinary skill in the art as found, for example, in Sambrook et al. (1989).

In accordance with the present invention, DNA molecules comprising $\Delta 6$ -desaturase genes have been
10 isolated. More particularly, a 3.588 kilobase (kb) DNA comprising a $\Delta 6$ -desaturase gene has been isolated from the cyanobacteria Synechocystis. The nucleotide sequence of the 3.588 kb DNA was determined and is shown in SEQ ID NO:1. Open reading frames defining
15 potential coding regions are present from nucleotide 317 to 1507 and from nucleotide 2002 to 3081. To define the nucleotides responsible for encoding $\Delta 6$ -desaturase, the 3.588 kb fragment that confers $\Delta 6$ -desaturase activity is cleaved into two subfragments,
20 each of which contains only one open reading frame. Fragment ORF1 contains nucleotides 1 through 1704, while fragment ORF2 contains nucleotides 1705 through 3588. Each fragment is subcloned in both forward and reverse orientations into a conjugal expression vector
25 (AM542, Wolk et al. [1984] Proc. Natl. Acad. Sci. USA 81, 1561) that contains a cyanobacterial carboxylase promoter. The resulting constructs (i.e. ORF1(F), ORF1(R), ORF2(F) and ORF2(R)) are conjugated to wild-type Anabaena PCC 7120 by standard methods (see, for
30 example, Wolk et al. (1984) Proc. Natl. Acad. Sci. USA 81, 1561). Conjugated cells of Anabaena are

1 identified as Neo^r green colonies on a brown
background of dying non-conjugated cells after two
weeks of growth on selective media (standard mineral
media BG11N + containing 30µg/ml of neomycin according
5 to Rippka et al., (1979) J. Gen Microbiol. 111, 1).
The green colonies are selected and grown in selective
liquid media (BG11N + with 15µg/ml neomycin). Lipids
are extracted by standard methods (e.g. Dahmer et al.,
(1989) Journal of American Oil Chemical Society 66,
10 543) from the resulting transconjugants containing the
forward and reverse oriented ORF1 and ORF2 constructs.
For comparison, lipids are also extracted from wild-
type cultures of Anabaena and Synechocystis. The
fatty acid methyl esters are analyzed by gas liquid
15 chromatography (GLC), for example with a Tracor-560
gas liquid chromatograph equipped with a hydrogen
flame ionization detector and a capillary column. The
results of GLC analysis are shown in Table 1.

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1 Table 1: Occurrence of C18 fatty acids in wild-type
and transgenic cyanobacteria

	SOURCE	18:0	18:1	18:2	γ 18:3	α 18:3	18:4
5	Anabaena (wild type)	+	+	+	-	+	-
	Anabaena + ORF1 (F)	+	+	+	-	+	-
	Anabaena + ORF1 (R)	+	+	+	-	+	-
	Anabaena + ORF2 (F)	+	+	+	+	+	+
10	Anabaena + ORF2 (R)	+	+	+	-	+	-
	Synechocystis (wild type)	+	+	+	+	-	-

As assessed by GLC analysis, GLA deficient
 15 Anabaena gain the function of GLA production when the
 construct containing ORF2 in forward orientation is
 introduced by transconjugation. Transconjugants
 containing constructs with ORF2 in reverse orientation
 to the carboxylase promoter, or ORF1 in either
 20 orientation, show no GLA production. This analysis
 demonstrates that the single open reading frame (ORF2)
 within the 1884 bp fragment encodes Δ 6-desaturase.
 The 1884 bp fragment is shown as SEQ ID NO:3. This is
 substantiated by the overall similarity of the
 25 hydropathy profiles between Δ 6-desaturase and Δ 12-
 desaturase [Wada et al. (1990) Nature 347] as shown in
 Fig. 1 as (A) and (B), respectively.

Also in accordance with the present
 invention, a cDNA comprising a Δ 6-desaturase gene from
 30 borage (Borago officinalis) has been isolated. The
 nucleotide sequence of the 1.685 kilobase (kb) cDNA

1 was determined and is shown in Fig. 5A (SEQ ID NO: 4).
The ATG start codon and stop codon are underlined.
The amino acid sequence corresponding to the open
reading frame in the borage delta 6-desaturase is
5 shown in Fig. 5B (SEQ ID NO: 5).

Isolated nucleic acids encoding $\Delta 6$ -
desaturase can be identified from other GLA-producing
organisms by the gain of function analysis described
above, or by nucleic acid hybridization techniques
10 using the isolated nucleic acid which encodes
Synechocystis or borage $\Delta 6$ -desaturase as a
hybridization probe. Both genomic and cDNA cloning
methods are known to the skilled artisan and are
contemplated by the present invention. The
15 hybridization probe can comprise the entire DNA
sequence disclosed as SEQ. ID NO:1 or SEQ. ID NO:4, or
a restriction fragment or other DNA fragment thereof,
including an oligonucleotide probe. Methods for
cloning homologous genes by cross-hybridization are
20 known to the ordinarily skilled artisan and can be
found, for example, in Sambrook (1989) and Beltz et
al. (1983) Methods in Enzymology 100, 266.

In another method of identifying a delta 6-
desaturase gene from an organism producing GLA, a cDNA
25 library is made from poly-A⁺ RNA isolated from
polysomal RNA. In order to eliminate hyper-abundant
expressed genes from the cDNA population, cDNAs or
fragments thereof corresponding to hyper-abundant
cDNAs genes are used as hybridization probes to the
30 cDNA library. Non hybridizing plaques are excised and
the resulting bacterial colonies are used to inoculate

1 liquid cultures and sequenced. For example, as a
means of eliminating other seed storage protein cDNAs
from a cDNA library made from borage polysomal RNA,
cDNAs corresponding to abundantly expressed seed
5 storage proteins are first hybridized to the cDNA
library. The "subtracted" DNA library is then used to
generate expressed sequence tags (ESTs) and such tags
are used to scan a data base such as GenBank to
identify potential desaturates.

10 Transgenic organisms which gain the function
of GLA production by introduction of DNA encoding Δ -
desaturase also gain the function of
octadecatetraenoic acid ($18:4^{6,9,12,15}$) production.
Octadecatetraenoic acid is present normally in fish
15 oils and in some plant species of the Boraginaceae
family (Craig et al. [1964] J. Amer. Oil Chem. Soc.
41, 209-211; Gross et al. [1976] Can. J. Plant Sci.
56, 659-664). In the transgenic organisms of the
present invention, octadecatetraenoic acid results
20 from further desaturation of α -linolenic acid by $\Delta 6$ -
desaturase or desaturation of GLA by $\Delta 15$ -desaturase.

The 359 amino acids encoded by ORF2, i.e.
the open reading frame encoding Synechocystis $\Delta 6$ -
desaturase, are shown as SEQ. ID NO:2. The open
25 reading frame encoding the borage $\Delta 6$ -desaturase is
shown in SEQ ID NO: 5. The present invention further
contemplates other nucleotide sequences which encode
the amino acids of SEQ ID NO:2 and SEQ ID NO: 5. It
is within the ken of the ordinarily skilled artisan to
30 identify such sequences which result, for example,
from the degeneracy of the genetic code. Furthermore,

1 one of ordinary skill in the art can determine, by the
gain of function analysis described hereinabove,
smaller subfragments of the fragments containing the
open reading frames which encode $\Delta 6$ -desaturases.

5 The present invention contemplates any such
polypeptide fragment of $\Delta 6$ -desaturase and the nucleic
acids therefor which retain activity for converting LA
to GLA.

In another aspect of the present invention,
10 a vector containing a nucleic acid of the present
invention or a smaller fragment containing the
promoter, coding sequence and termination region of a
 $\Delta 6$ -desaturase gene is transferred into an organism,
for example, cyanobacteria, in which the $\Delta 6$ -desaturase
15 promoter and termination regions are functional.
Accordingly, organisms producing recombinant $\Delta 6$ -
desaturase are provided by this invention. Yet
another aspect of this invention provides isolated $\Delta 6$ -
desaturase, which can be purified from the recombinant
20 organisms by standard methods of protein purification.
(For example, see Ausubel et al. [1987] Current
Protocols in Molecular Biology, Green Publishing
Associates, New York).

Vectors containing DNA encoding $\Delta 6$ -
25 desaturase are also provided by the present invention.
It will be apparent to one of ordinary skill in the
art that appropriate vectors can be constructed to
direct the expression of the $\Delta 6$ -desaturase coding
sequence in a variety of organisms. Replicable
expression vectors are particularly preferred.
30 Replicable expression vectors as described herein are

1 DNA or RNA molecules engineered for controlled
expression of a desired gene, i.e. the $\Delta 6$ -desaturase
gene. Preferably the vectors are plasmids,
bacteriophages, cosmids or viruses. Shuttle vectors,
5 e.g. as described by Wolk et al. (1984) Proc. Natl.
Acad. Sci. USA, 1561-1565 and Bustos et al. (1991) J.
Bacteriol. 174, 7525-7533, are also contemplated in
accordance with the present invention. Sambrook et
al. (1989), Goeddel, ed. (1990) Methods in Enzymology
10 185 Academic Press, and Perbal (1988) A Practical
Guide to Molecular Cloning, John Wiley and Sons, Inc.,
provide detailed reviews of vectors into which a
nucleic acid encoding the present $\Delta 6$ -desaturase can be
inserted and expressed. Such vectors also contain
15 nucleic acid sequences which can effect expression of
nucleic acids encoding $\Delta 6$ -desaturase. Sequence
elements capable of effecting expression of a gene
product include promoters, enhancer elements, upstream
activating sequences, transcription termination
20 signals and polyadenylation sites. Both constitutive
and tissue specific promoters are contemplated. For
transformation of plant cells, the cauliflower mosaic
virus (CaMV) 35S promoter and promoters which are
regulated during plant seed maturation are of
25 particular interest. All such promoter and
transcriptional regulatory elements, singly or in
combination, are contemplated for use in the present
replicable expression vectors and are known to one of
ordinary skill in the art. The CaMV 35S promoter is
30 described, for example, by Restrepo et al. (1990)

1 Plant Cell 2, 987. Genetically engineered and mutated regulatory sequences are also contemplated.

The ordinarily skilled artisan can determine vectors and regulatory elements suitable for
5 expression in a particular host cell. For example, a vector comprising the promoter from the gene encoding the carboxylase of Anabaena operably linked to the coding region of $\Delta 6$ -desaturase and further operably linked to a termination signal from Synechocystis is
10 appropriate for expression of $\Delta 6$ -desaturase in cyanobacteria. "Operably linked" in this context means that the promoter and terminator sequences effectively function to regulate transcription. As a further example, a vector appropriate for expression
15 of $\Delta 6$ -desaturase in transgenic plants can comprise a seed-specific promoter sequence derived from helianthinin, napin, or glycinin operably linked to the $\Delta 6$ -desaturase coding region and further operably linked to a seed termination signal or the nopaline
20 synthase termination signal. As a still further example, a vector for use in expression of $\Delta 6$ -desaturase in plants can comprise a constitutive promoter or a tissue specific promoter operably linked to the $\Delta 6$ -desaturase coding region and further
25 operably linked to a constitutive or tissue specific terminator or the nopaline synthase termination signal.

In particular, the helianthinin regulatory elements disclosed in applicant's copending U.S.
30 Application Serial No. 682,354, filed April 8, 1991 and incorporated herein by reference, are contemplated

1 as promoter elements to direct the expression of the
Δ6-desaturase of the present invention.

Modifications of the nucleotide sequences or
regulatory elements disclosed herein which maintain
5 the functions contemplated herein are within the scope
of this invention. Such modifications include
insertions, substitutions and deletions, and
specifically substitutions which reflect the
degeneracy of the genetic code.

10 Standard techniques for the construction of
such hybrid vectors are well-known to those of
ordinary skill in the art and can be found in
references such as Sambrook et al. (1989), or any of
the myriad of laboratory manuals on recombinant DNA
15 technology that are widely available. A variety of
strategies are available for ligating fragments of
DNA, the choice of which depends on the nature of the
termini of the DNA fragments. It is further
contemplated in accordance with the present invention
20 to include in the hybrid vectors other nucleotide
sequence elements which facilitate cloning, expression
or processing, for example sequences encoding signal
peptides, a sequence encoding KDEL, which is required
for retention of proteins in the endoplasmic reticulum
25 or sequences encoding transit peptides which direct
Δ6-desaturase to the chloroplast. Such sequences are
known to one of ordinary skill in the art. An
optimized transit peptide is described, for example,
by Van den Broeck et al. (1985) Nature 313, 358.
30 Prokaryotic and eukaryotic signal sequences are

1 disclosed, for example, by Michaelis et al. (1982)
Ann. Rev. Microbiol. 36, 425.

A further aspect of the instant invention provides organisms other than cyanobacteria or plants
5 which contain the DNA encoding the $\Delta 6$ -desaturase of the present invention. The transgenic organisms contemplated in accordance with the present invention include bacteria, cyanobacteria, fungi, and plants and animals. The isolated DNA of the present invention
10 can be introduced into the host by methods known in the art, for example infection, transfection, transformation or transconjugation. Techniques for transferring the DNA of the present invention into such organisms are widely known and provided in
15 references such as Sambrook et al. (1989).

A variety of plant transformation methods are known. The $\Delta 6$ -desaturase gene can be introduced into plants by a leaf disk transformation-regeneration procedure as described by Horsch et al. (1985) Science
20 227, 1229. Other methods of transformation, such as protoplast culture (Horsch et al. (1984) Science 223, 496; DeBlock et al. (1984) EMBO J. 2, 2143; Barton et al. (1983) Cell 32, 1033) can also be used and are within the scope of this invention. In a preferred
25 embodiment plants are transformed with Agrobacterium-derived vectors. However, other methods are available to insert the $\Delta 6$ -desaturase genes of the present invention into plant cells. Such alternative methods include biolistic approaches (Klein et al. (1987)
30 Nature 327, 70), electroporation, chemically-induced DNA uptake, and use of viruses or pollen as vectors.

1 When necessary for the transformation
method, the $\Delta 6$ -desaturase genes of the present
invention can be inserted into a plant transformation
vector, e.g. the binary vector described by Bevan
5 (1984) Nucleic Acids Res. 12, 8111. Plant
transformation vectors can be derived by modifying the
natural gene transfer system of Agrobacterium
tumefaciens. The natural system comprises large Ti
(tumor-inducing)-plasmids containing a large segment,
10 known as T-DNA, which is transferred to transformed
plants. Another segment of the Ti plasmid, the vir
region, is responsible for T-DNA transfer. The T-DNA
region is bordered by terminal repeats. In the
modified binary vectors the tumor-inducing genes have
15 been deleted and the functions of the vir region are
utilized to transfer foreign DNA bordered by the T-DNA
border sequences. The T-region also contains a
selectable marker for antibiotic resistance, and a
multiple cloning site for inserting sequences for
20 transfer. Such engineered strains are known as
"disarmed" A. tumefaciens strains, and allow the
efficient transformation of sequences bordered by the
T-region into the nuclear genomes of plants.

Surface-sterilized leaf disks are inoculated
25 with the "disarmed" foreign DNA-containing A.
tumefaciens, cultured for two days, and then
transferred to antibiotic-containing medium.
Transformed shoots are selected after rooting in
medium containing the appropriate antibiotic,
30 transferred to soil and regenerated.

1 Another aspect of the present invention
provides transgenic plants or progeny of these plants
containing the isolated DNA of the invention. Both
monocotyledenous and dicotyledenous plants are
5 contemplated. Plant cells are transformed with the
isolated DNA encoding $\Delta 6$ -desaturase by any of the
plant transformation methods described above. The
transformed plant cell, usually in a callus culture or
leaf disk, is regenerated into a complete transgenic
10 plant by methods well-known to one of ordinary skill
in the art (e.g. Horsch et al. (1985) Science 227,
1129). In a preferred embodiment, the transgenic
plant is sunflower, oil seed rape, maize, tobacco,
peanut or soybean. Since progeny of transformed
15 plants inherit the DNA encoding $\Delta 6$ -desaturase, seeds
or cuttings from transformed plants are used to
maintain the transgenic plant line.

The present invention further provides a
method for providing transgenic plants with an
20 increased content of GLA. This method includes
introducing DNA encoding $\Delta 6$ -desaturase into plant
cells which lack or have low levels of GLA but contain
LA, and regenerating plants with increased GLA content
from the transgenic cells. In particular,
25 commercially grown crop plants are contemplated as the
transgenic organism, including, but not limited to,
sunflower, soybean, oil seed rape, maize, peanut and
tobacco.

The present invention further provides a
30 method for providing transgenic organisms which
contain GLA. This method comprises introducing DNA

1 encoding $\Delta 6$ -desaturase into an organism which lacks or
has low levels of GLA, but contains LA. In another
embodiment, the method comprises introducing one or
more expression vectors which comprise DNA encoding
5 $\Delta 12$ -desaturase and $\Delta 6$ -desaturase into organisms which
are deficient in both GLA and LA. Accordingly,
organisms deficient in both LA and GLA are induced to
produce LA by the expression of $\Delta 12$ -desaturase, and
GLA is then generated due to the expression of $\Delta 6$ -
10 desaturase. Expression vectors comprising DNA
encoding $\Delta 12$ -desaturase, or $\Delta 12$ -desaturase and $\Delta 6$ -
desaturase, can be constructed by methods of
recombinant technology known to one of ordinary skill
in the art (Sambrook et al., 1989) and the published
15 sequence of $\Delta 12$ -desaturase (Wada et al [1990] Nature
(London) 347, 200-203. In addition, it has been
discovered in accordance with the present invention
that nucleotides 2002-3081 of SEQ. ID NO:1 encode
cyanobacterial $\Delta 12$ -desaturase. Accordingly, this
20 sequence can be used to construct the subject
expression vectors. In particular, commercially grown
crop plants are contemplated as the transgenic
organism, including, but not limited to, sunflower,
soybean, oil seed rape, maize, peanut and tobacco.
25 The present invention is further directed to
a method of inducing chilling tolerance in plants.
Chilling sensitivity may be due to phase transition of
lipids in cell membranes. Phase transition
temperature depends upon the degree of unsaturation of
30 fatty acids in membrane lipids, and thus increasing
the degree of unsaturation, for example by introducing

1 Δ^6 -desaturase to convert LA to GLA, can induce or
improve chilling resistance. Accordingly, the present
method comprises introducing DNA encoding Δ^6 -
desaturase into a plant cell, and regenerating a plant
5 with improved chilling resistance from said
transformed plant cell. In a preferred embodiment,
the plant is a sunflower, soybean, oil seed rape,
maize, peanut or tobacco plant.

The following examples further illustrate
10 the present invention.

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EXAMPLE 1

Strains and Culture Conditions

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5 Synechocystis (PCC 6803, ATCC 27184),
 Anabaena (PCC 7120, ATCC 27893) and Synechococcus (PCC
 7942, ATCC 33912) were grown photoautotrophically at
 30°C in BG11N+ medium (Rippka et al. [1979] J. Gen.
 Microbiol. 111, 1-61) under illumination of
 incandescent lamps
10 (60 μ E.m⁻².S⁻¹). Cosmids and plasmids were selected and
 propagated in Escherichia coli strain DH5 α on LB
 medium supplemented with antibiotics at standard
 concentrations as described by Maniatis et al. (1982)
 Molecular Cloning: A Laboratory Manual, Cold Spring
15 Harbor Laboratory, Cold Spring, New York.

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EXAMPLE 2

Construction of Synechocystis Cosmid Genomic Library

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5 Total genomic DNA from Synechocystis (PCC 6803) was partially digested with Sau3A and fractionated on a sucrose gradient (Ausubel et al. [1987] Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, New York). Fractions containing 30 to 40 kb DNA fragments
10 were selected and ligated into the dephosphorylated BamHI site of the cosmid vector, pDUCA7 (Buikema et al. [1991] J. Bacteriol. 173, 1879-1885). The ligated DNA was packaged in vitro as described by Ausubel et al. (1987), and packaged phage were propagated in E.
15 coli DH5 α containing the AvaI and Eco4711 methylase helper plasmid, pRL528 as described by Buikema et al. (1991). A total of 1152 colonies were isolated randomly and maintained individually in twelve 96-well microtiter plates.

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EXAMPLE 3

Gain-of-Function Expression of GLA in Anabaena

Anabaena (PCC 7120), a filamentous
5 cyanobacterium, is deficient in GLA but contains
significant amounts of linoleic acid, the precursor
for GLA (Figure 2; Table 2). The Synechocystis cosmid
library described in Example 2 was conjugated into
10 Anabaena (PCC 7120) to identify transconjugants that
produce GLA. Anabaena cells were grown to mid-log
phase in BG11N+ liquid medium and resuspended in the
same medium to a final concentration of approximately
2x10⁸ cells per ml. A mid-log phase culture of E.
15 coli RP4 (Burkardt et al. [1979] J. Gen. Microbiol.
114, 341-348) grown in LB containing ampicillin was
washed and resuspended in fresh LB medium. Anabaena
and RP4 were then mixed and spread evenly on BG11N+
plates containing 5% LB. The cosmid genomic library
20 was replica plated onto LB plates containing 50 µg/ml
kanamycin and 17.5 µg/ml chloramphenicol and was
subsequently patched onto BG11N+ plates containing
Anabaena and RP4. After 24 hours of incubation at
30°C, 30 µg/ml of neomycin was underlaid; and
incubation at 30°C was continued until transconjugants
25 appeared.

Individual transconjugants were isolated
after conjugation and grown in 2 ml BG11N+ liquid
medium with 15 µg/ml neomycin. Fatty acid methyl
esters were prepared from wild type cultures and
30 cultures containing pools of ten transconjugants as
follows. Wild type and transgenic cyanobacterial

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1 cultures were harvested by centrifugation and washed
twice with distilled water. Fatty acid methyl esters
were extracted from these cultures as described by
Dahmer et al. (1989) J. Amer. Oil. Chem. Soc. 66, 543-
5 548 and were analyzed by Gas Liquid Chromatography
(GLC) using a Tracor-560 equipped with a hydrogen
flame ionization detector and capillary column (30 m x
0.25 mm bonded FSOT Superox II, Alltech Associates
Inc., IL). Retention times and co-chromatography of
10 standards (obtained from Sigma Chemical Co.) were used
for identification of fatty acids. The average fatty
acid composition was determined as the ratio of peak
area of each C18 fatty acid normalized to an internal
standard.

15 Representative GLC profiles are shown in
Fig. 2. C18 fatty acid methyl esters are shown.
Peaks were identified by comparing the elution times
with known standards of fatty acid methyl esters and
were confirmed by gas chromatography-mass
20 spectrometry. Panel A depicts GLC analysis of fatty
acids of wild type Anabaena. The arrow indicates the
migration time of GLA. Panel B is a GLC profile of
fatty acids of transconjugants of Anabaena with
pAM542+1.8F. Two GLA producing pools (of 25 pools
25 representing 250 transconjugants) were identified that
produced GLA. Individual transconjugants of each GLA
positive pool were analyzed for GLA production; two
independent transconjugants, AS13 and AS75, one from
each pool, were identified which expressed significant
30 levels of GLA and which contained cosmids, cSy13 and
cSy75, respectively (Figure 3). The cosmids overlap

1 in a region approximately 7.5 kb in length. A 3.5 kb
NheI fragment of cSy75 was recloned in the vector
pDUCA7 and transferred to Anabaena resulting in gain-
of-function expression of GLA (Table 2).

5 Two NheI/Hind III subfragments (1.8 and 1.7
kb) of the 3.5 kb Nhe I fragment of cSy75-3.5 were
subcloned into "pBLUESCRIPT" (Stratagene) (Figure 3)
for sequencing. Standard molecular biology techniques
were performed as described by Maniatis et al. (1982)
10 and Ausubel et al. (1987). Dideoxy sequencing (Sanger
et al. [1977] Proc. Natl. Acad. Sci. USA 74, 5463-
5467) of pBS1.8 was performed with "SEQUENASE" (United
States Biochemical) on both strands by using specific
oligonucleotide primers synthesized by the Advanced
15 DNA Technologies Laboratory (Biology Department, Texas
A & M University). DNA sequence analysis was done
with the GCG (Madison, WI) software as described by
Devereux et al. (1984) Nucleic Acids Res. 12, 387-395.

Both NheI/HindIII subfragments were
20 transferred into a conjugal expression vector, AM542,
in both forward and reverse orientations with respect
to a cyanobacterial carboxylase promoter and were
introduced into Anabaena by conjugation.
Transconjugants containing the 1.8 kb fragment in the
25 forward orientation (AM542-1.8F) produced significant
quantities of GLA and octadecatetraenoic acid (Figure
2; Table 2). Transconjugants containing other
constructs, either reverse oriented 1.8 kb fragment or
forward and reverse oriented 1.7 kb fragment, did not
30 produce detectable levels of GLA (Table 2).

1 Figure 2 compares the C18 fatty acid profile
of an extract from wild type Anabaena (Figure 2A) with
that of transgenic Anabaena containing the 1.8 kb
fragment of cSy75-3.5 in the forward orientation
5 (Figure 2B). GLC analysis of fatty acid methyl esters
from AM542-1.8F revealed a peak with a retention time
identical to that of authentic GLA standard. Analysis
of this peak by gas chromatography-mass spectrometry
(GC-MS) confirmed that it had the same mass
10 fragmentation pattern as a GLA reference sample.
Transgenic Anabaena with altered levels of
polyunsaturated fatty acids were similar to wild type
in growth rate and morphology.

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1 Table 2 Composition of C18 Fatty Acids in Wild Type
and Transgenic Cyanobacteria

5	Strain	Fatty Acid (%)					
		18:0	18:1	18:2	18.3(α)	18.3(γ)	18.4
	Wild Type						
10	<i>Synechocystis</i> (sp. PCC6803)	13.6	4.5	54.5	-	27.3	-
	<i>Anabaena</i> (sp. PCC7120)	2.9	24.8	37.1	35.2	-	-
15	<i>Synechococcus</i> (sp. PCC7942)	20.6	79.4	-	-	-	-
	<i>Anabaena</i> Transconjugants						
	cSy75	3.8	24.4	22.3	9.1	27.9	12.5
	cSy75-3.5	4.3	27.6	18.1	3.2	40.4	6.4
20	pAM542 - 1.8F	4.2	13.9	12.1	19.1	25.4	25.4
	pAM542 - 1.8R	7.7	23.1	38.4	30.8	-	-
	pAM542 - 1.7F	2.8	27.8	36.1	33.3	-	-
	pAM542 - 1.7R	2.8	25.4	42.3	29.6	-	-
	<i>Synechococcus</i> Transformants						
25	pAM854	27.8	72.2	-	-	-	-
	pAM854 -Δ ¹²	4.0	43.2	46.0	-	-	-
	pAM854 -Δ ⁶	18.2	81.8	-	-	-	-
	pAM854 -Δ ⁶ &Δ ¹²	42.7	25.3	19.5	-	16.5	-
30	18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3(α), linolenic acid; 18:3(γ), γ-linolenic acid; 18:4, octadecatetraenoic acid						

EXAMPLE 4

Transformation of Synechococcus
with $\Delta 6$ and $\Delta 12$ Desaturase Genes

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5 A third cosmid, cSy7, which contains a $\Delta 12$ -
desaturase gene, was isolated by screening the
Synechocystis genomic library with a oligonucleotide
synthesized from the published Synechocystis $\Delta 12$ -
desaturase gene sequence (Wada et al. [1990] Nature
10 (London) 347, 200-203). A 1.7 kb AvaI fragment from
this cosmid containing the $\Delta 12$ -desaturase gene was
identified and used as a probe to demonstrate that
cSy13 not only contains a $\Delta 6$ -desaturase gene but also
a $\Delta 12$ -desaturase gene (Figure 3). Genomic Southern
15 blot analysis further showed that both the $\Delta 6$ -and $\Delta 12$ -
desaturase genes are unique in the Synechocystis
genome so that both functional genes involved in C18
fatty acid desaturation are linked closely in the
Synechocystis genome.

20 The unicellular cyanobacterium Synechococcus
(PCC 7942) is deficient in both linoleic acid and
GLA(3). The $\Delta 12$ and $\Delta 6$ -desaturase genes were cloned
individually and together into pAM854 (Bustos et al.
[1991] J. Bacteriol. 174, 7525-7533), a shuttle vector
that contains sequences necessary for the integration
25 of foreign DNA into the genome of Synechococcus
(Golden et al. [1987] Methods in Enzymol. 153, 215-
231). Synechococcus was transformed with these gene
constructs and colonies were selected. Fatty acid
methyl esters were extracted from transgenic
30 Synechococcus and analyzed by GLC.

1 Table 2 shows that the principal fatty acids
of wild type Synechococcus are stearic acid (18:0) and
oleic acid (18:1). Synechococcus transformed with
pAM854- Δ 12 expressed linoleic acid (18:2) in addition
5 to the principal fatty acids. Transformants with
pAM854- Δ 6 and Δ 12 produced both linoleate and GLA
(Table 1). These results indicated that Synechococcus
containing both Δ 12- and Δ 6-desaturase genes has
gained the capability of introducing a second double
10 bond at the Δ 12 position and a third double bond at
the Δ 6 position of C18 fatty acids. However, no
changes in fatty acid composition was observed in the
transformant containing pAM854- Δ 6, indicating that in
the absence of substrate synthesized by the Δ 12
15 desaturase, the Δ 6-desaturase is inactive. This
experiment further confirms that the 1.8 kb
NheI/HindIII fragment (Figure 3) contains both coding
and promoter regions of the Synechocystis Δ 6-
desaturase gene. Transgenic Synechococcus with
20 altered levels of polyunsaturated fatty acids were
similar to wild type in growth rate and morphology.

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EXAMPLE 5

Nucleotide Sequence of $\Delta 6$ -Desaturase

1 The nucleotide sequence of the 1.8 kb
5 fragment of cSy75-3.5 including the functional $\Delta 6$ -
desaturase gene was determined. An open reading frame
encoding a polypeptide of 359 amino acids was
identified (Figure 4). A Kyte-Doolittle hydropathy
analysis (Kyte et al. [1982] J. Mol. Biol. 157, 105-
10 132) identified two regions of hydrophobic amino acids
that could represent transmembrane domains (Figure
1A); furthermore, the hydropathic profile of the $\Delta 6$ -
desaturase is similar to that of the $\Delta 12$ -desaturase
gene (Figure 1B; Wada et al.) and $\Delta 9$ -desaturases
15 (Thiede et al. [1986] J. Biol. Chem. 261, 13230-
13235). However, the sequence similarity between the
Synechocystis $\Delta 6$ - and $\Delta 12$ -desaturases is less than 40%
at the nucleotide level and approximately 18% at the
amino acid level.

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EXAMPLE 6

Transfer of Cyanobacterial Δ^6 -Desaturase into Tobacco

The cyanobacterial Δ^6 -desaturase gene was mobilized into a plant expression vector and transferred to tobacco using Agrobacterium mediated gene transfer techniques. To ensure that the transferred desaturase is appropriately expressed in leaves and developing seeds and that the desaturase gene product is targeted to the endoplasmic reticulum or the chloroplast, various expression cassettes with Synechocystis Δ -desaturase open reading frame (ORF) were constructed. Components of these cassettes include: (i) a 35S promoter or seed specific promoter derived from the sunflower helianthinin gene to drive Δ^6 -desaturase gene expression in all plant tissues or only in developing seeds respectively, (ii) a putative signal peptide either from carrot extensin gene or sunflower helianthinin gene to target newly synthesized Δ^6 -desaturase into the ER, (iii) an ER lumen retention signal sequence (KDEL) at the COOH-terminal of the Δ^6 -desaturase ORF, and (iv) an optimized transit peptide to target Δ^6 desaturase into the chloroplast. The 35S promoter is a derivative of pRTL2 described by Restrepo et al. (1990). The optimized transit peptide sequence is described by Van de Broeck et al. (1985). The carrot extensin signal peptide is described by Chen et al (1985) EMBO J. 9, 2145.

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Transgenic tobacco plants were produced containing a chimeric cyanobacterial desaturase gene,

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1 comprised of the Synechocystis Δ^6 desaturase gene fused
to an endoplasmic reticulum retention sequence (KDEL)
and extensin signal peptide driven by the CaMV 35S
promoter. PCR amplifications of transgenic tobacco
5 genomic DNA indicate that the Δ^6 desaturase gene was
incorporated into the tobacco genome. Fatty acid
methyl esters of leaves of these transgenic tobacco
plants were extracted and analyzed by Gas Liquid
Chromatography (GLC). These transgenic tobacco
10 accumulated significant amounts of GLA (Figure 4).
Figure 4 shows fatty acid methyl esters as determined
by GLC. Peaks were identified by comparing the
elution times with known standards of fatty acid
methyl ester. Accordingly, cyanobacterial genes
15 involved in fatty acid metabolism can be used to
generate transgenic plants with altered fatty acid
compositions.

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EXAMPLE 7

Construction of Borage cDNA library

Membrane bound polysomes were isolated from
5 borage seeds 12 days post pollination (12 DPP) using
the protocol established for peas by Larkins and
Davies (1975 Plant Phys. 55:749-756). RNA was
extracted from the polysomes as described by Mechler
(1987 Methods in Enzymology 152:241-248, Academic
10 Press).

Poly-A+ RNA was isolated from the membrane
bound polysomal RNA by use of Oligotex-dT beads
(Qiagen). Corresponding cDNA was made using
Stratagene's ZAP cDNA synthesis kit. The cDNA library
15 was constructed in the lambda ZAP II vector
(Stratagene) using the lambda ZAP II vector kit. The
primary library was packaged in Gigapack II Gold
packaging extract (Stratagene). The library was used
to generate expressed sequence tags (ESTs), and
20 sequences corresponding to the tags were used to scan
the GenBank database.

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EXAMPLE 8

Hybridization Protocol

Hybridization probes for screening the
borage cDNA library were generated by using random
primed DNA synthesis as described by Ausubel et al
(1994 Current Protocols in Molecular Biology, Wiley
Interscience, N.Y.) and corresponded to previously
identified abundantly expressed seed storage protein
cDNAs. Unincorporated nucleotides were removed by use
of a G-50 spin column (Boehringer Mannheim). Probe was
denatured for hybridization by boiling in a water bath
for 5 minutes, then quickly cooled on ice. Filters
for hybridization were prehybridized at 60°C for 2-4
hours in prehybridization solution (6XSSC [Maniatis et
al 1984 Molecular Cloning A Laboratory Manual, Cold
Spring Harbor Laboratory], 1X Denharts Solution, 0.05%
sodium pyrophosphate, 100 µg/ml denatured salmon sperm
DNA). Denatured probe was added to the hybridization
solution (6X SSC, 1X Denharts solution, 0.05% sodium
pyrophosphate, 100 µg/ml denatured salmon sperm DNA)
and incubated at 60°C with agitation overnight.
Filters were washed in 4x, 2x, and 1x SET washes for
15 minutes each at 60°C. A 20X SET stock solution is
3M NaCl, 0.4 M Tris base, 20 mM Na₂EDTA-2H₂O. The 4X
SET wash was 4X SET, 12.5 mM PO₄, pH 6.8 and 0.2% SDS.
The 2X SET wash was 2X SET, 12.5 mM PO₄, pH 6.8 and
0.2% SDS. The 1X SET wash was 1X SET, 12.5 mM PO₄, pH
6.8 and 0.2% SDS. Filters were allowed to air dry and
were then exposed to X-ray film for 24 hours with
intensifying screens at -80°C.

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EXAMPLE 9

Random sequencing of cDNAs from a borage seed
(12 DPP) membrane-bound polysomal library

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The borage cDNA library was plated at low density (500 pfu on 150 mm petri dishes). Highly prevalent seed storage protein cDNAs were "subtracted" by screening with the previously identified corresponding cDNAs. Non-hybridizing plaques were excised using Stratagene's excision protocol and reagents. Resulting bacterial colonies were used to inoculate liquid cultures and were either sequenced manually or by an ABI automated sequencer. Each cDNA was sequenced once and a sequence tag generated from 200-300 base pairs. All sequencing was performed by cycle sequencing (Epicentre). Over 300 ESTs were generated. Each sequence tag was compared to GenBank database by BLASTX computer program and a number of lipid metabolism genes, including the $\Delta 6$ -desaturase were identified.

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Database searches with a cDNA clone designated mbp-65 using BLASTX with the GenBank database resulted in a significant match to the Synechocystis $\Delta 6$ -desaturase. It was determined however, that this clone was not a full length cDNA. A full length cDNA was isolated using mbp-65 to screen the borage membrane-bound polysomal library. The sequence of the isolated cDNA was determined (Fig. 5A, SEQ ID NO:4) and the protein sequence of the open reading frame (Fig. 5B, SEQ ID NO:5) was compared to other known desaturases using Geneworks

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1 (IntelligGenetics) protein alignment program (Fig. 2).
This alignment indicated that the cDNA was the borage
Δ6-desaturase gene.

Although similar to other known plant
5 desaturases, the borage delta 6-desaturase is distinct
as indicated in the dendrogram shown in Fig. 6.
Furthermore, comparison of the amino acid sequences
characteristic of desaturases, particularly those
proposed to be involved in metal binding (metal box 1
10 and metal box 2), illustrates the differences between
the borage delta 6-desaturase and other plant
desaturases (Table 3).

The borage delta 6-desaturase is
distinguished from the cyanobacterial form not only in
15 over all sequence (Fig. 6) but also in the lipid box,
metal box 1 and metal box 2 amino acid motifs (Table
3). As Table 3 indicates, all three motifs are novel
in sequence. Only the borage delta 6-desaturase metal
box 2 shown some relationship to the Synechocystis
20 delta-6 desaturase metal box 2.

In addition, the borage delta 6-desaturase
is also distinct from another borage desaturase gene,
the delta-12 desaturase. P1-81 is a full length cDNA
that was identified by EST analysis and shows high
25 similarity to the Arabidopsis delta-12 desaturase (Fad
2). A comparison of the lipid box, metal box 1 and
metal box 2 amino acid motifs (Table 3) in borage
delta 6 and delta-12 desaturases indicates that little
homology exists in these regions. The placement of
30 the two sequences in the dendrogram in Fig. 6
indicates how distantly related these two genes are.

Table 3. Comparison of common amino acid motifs in membrane-bound desaturases

Desaturase	Amino Acid Motif					
	Lipid Box		Metal Box 1		Metal Box 2	
Borage Δ^6	WIGHDAGH (SEQ. ID. NO: 6)	HNAHH (SEQ. ID. NO: 12)	FQIEHH (SEQ. ID. NO: 20)			
Synechocystis Δ^6	NVGHDANH (SEQ. ID. NO: 7)	HNYLHH (SEQ. ID. NO: 13)	HQVTHH (SEQ. ID. NO: 21)			
Arab. chloroplast Δ^{15}	VLGHDCGH (SEQ. ID. NO: 8)	HRTHH (SEQ. ID. NO: 14)	HVIHH (SEQ. ID. NO: 22)			
Rice Δ^{15}	VLGHDCGH (SEQ. ID. NO: 8)	HRTHH (SEQ. ID. NO: 14)	HVIHH (SEQ. ID. NO: 22)			
Glycine chloroplast Δ^{15}	VLGHDCGH (SEQ. ID. NO: 8)	HRTHH (SEQ. ID. NO: 14)	HVIHH (SEQ. ID. NO: 22)			
Arab. fad3 (Δ^{15})	VLGHDCGH (SEQ. ID. NO: 8)	HRTHH (SEQ. ID. NO: 14)	HVIHH (SEQ. ID. NO: 22)			
Brassica fad3 (Δ^{15})	VLGHDCGH (SEQ. ID. NO: 8)	HRTHH (SEQ. ID. NO: 14)	HVIHH (SEQ. ID. NO: 22)			
Borage Δ^{12} (Pl-81)*	VIAHECGH (SEQ. ID. NO: 9)	HRRHH (SEQ. ID. NO: 15)	HVAHH (SEQ. ID. NO: 23)			
Arab. fad2 (Δ^{17})	VIAHECGH (SEQ. ID. NO: 9)	HRRHH (SEQ. ID. NO: 15)	HVAHH (SEQ. ID. NO: 23)			
Arab. chloroplast Δ^{12}	VIGHDCAH (SEQ. ID. NO: 10)	HDRHH (SEQ. ID. NO: 16)	HIPHH (SEQ. ID. NO: 24)			
Glycine plastid Δ^{12}	VIGHDCAH (SEQ. ID. NO: 10)	HDRHH (SEQ. ID. NO: 16)	HIPHH (SEQ. ID. NO: 24)			
Spinach plastidial n-6	VIGHDCAH (SEQ. ID. NO: 10)	HDQHH (SEQ. ID. NO: 17)	HIPHH (SEQ. ID. NO: 24)			
Synechocystis Δ^{12}	VVGHDGCH (SEQ. ID. NO: 11)	HDHHH (SEQ. ID. NO: 18)	HIPHH (SEQ. ID. NO: 24)			
Anabaena Δ^{12}	VLGHDCGH (SEQ. ID. NO: 8)	HNHHH (SEQ. ID. NO: 19)	HVPHH (SEQ. ID. NO: 25)			

*Pl-81 is a full length cDNA which was identified by EST analysis and shows high similarity to the Arabidopsis Δ^{12} desaturase (fad2)

EXAMPLE 10

Construction of 222.1 Δ^6 NOS for transient
and expression

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5 The vector pBI221 (Jefferson et al. 1987
EMBO J. 6:3901-3907) was prepared for ligation by
digestion with BamHI and EcoICR I (Promega) which
excises the GUS coding region leaving the 35S promoter
and NOS terminator intact. The borage Δ^6 -desaturase
10 cDNA was excised from the Bluescript plasmid
(Stratagene) by digestion with BamHI and XhoI. The
XhoI end was made blunt by use of the Klenow fragment.
This fragment was then cloned into the BamHI/EcoICR I
sites of pBI221, yielding 221. Δ^6 NOS (Fig. 7). In
15 221. Δ^6 .NOS, the remaining portion (backbone) of the
restriction map depicted in Fig. 7 is pBI221.

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EXAMPLE 11

Construction of 121. Δ^6 .NOS for stable transformation

5 The vector pBI121 (Jefferson et al. 1987
EMBO J. 6:3901-3907) was prepared for ligation by
digestion with BamHI and EcoICR I (Promega) which
excises the GUS coding region leaving the 35S promoter
and NOS terminator intact. The borage Δ^6 -desaturase
cDNA was excised from the Bluescript plasmid
10 (Stratagene) by digestion with BamHI and XhoI. The
XhoI end was made blunt by use of the Klenow fragment.
This fragment was then cloned into the BamHI/EcoICR I
sites of pBI121, yielding 121.1 Δ^6 NOS (Fig. 7). In
121. Δ^6 .NOS, the remaining portion (backbone) of the
15 restriction map depicted in Fig. 7 is pBI121.

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EXAMPLE 12

Transient Expression

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5 All work involving protoplasts was performed
in a sterile hood. One ml of packed carrot suspension
cells were digested in 30 mls plasmolyzing solution
(25 g/l KCl, 3.5 g/l CaCl₂-H₂O, 10mM MES, pH 5.6 and
0.2 M mannitol) with 1% cellulase, 0.1% pectolyase,
and 0.1% dreisalase overnight, in the dark, at room
10 temperature. Released protoplasts were filtered
through a 150 μ m mesh and pelleted by centrifugation
(100x g, 5 min.) then washed twice in plasmolyzing
solution. Protoplasts were counted using a double
chambered hemocytometer. DNA was transfected into the
15 protoplasts by PEG treatment as described by Nunberg
and Thomas (1993 Methods in Plant Molecular Biology
and Biotechnology, B.R. Glick and J.E. Thompson, eds.
pp. 241-248) using 10⁶ protoplasts and 50-70 ug of
plasmid DNA (221. Δ 6.NOS). Protoplasts were cultured
20 in 5 mls of MS media supplemented with 0.2M mannitol
and 3 μ m 2,4-D for 48 hours in the dark with shaking.

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EXAMPLE 13

Stable transformation of tobacco

121.Δ⁶.NOS plasmid construction was used to
5 transform tobacco (*Nicotiana tabacum* cv. xanthi) via
Agrobacterium according to standard procedures (Horsh
et al., 1985 Science 227: 1229-1231; Bogue et al.,
1990 Mol. Gen. Genet. 221:49-57), except that initial
transformants were selected on 100 ug/ml kanamycin.

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EXAMPLE 14

Preparation and analysis of
fatty acid methyl esters (FAMES)

1 Tissue from transfected protoplasts and
5 transformed tobacco plants was frozen in liquid
nitrogen and lyophilized overnight. FAMES were
prepared as described by Dahmer et al (1989 J. Amer.
Oil Chem. Soc. 66:543-548). In some cases, the
solvent was evaporated again, and the FAMES were
10 resuspended in ethyl acetate and extracted once with
deionized water to remove any water soluble
contaminants. The FAMES were analyzed by gas
chromatography (GC) on a J&W Scientific DB-wax column
(30 m length, 0.25 mm ID, 0.25 μ m film).

15 An example of a transient assay is shown in
Fig. 8 which represents three independent
transfections pooled together. The addition of the
borage $\Delta 6$ -desaturase cDNA corresponds with the
appearance of gamma linolenic acid (GLA) which is one
20 of the possible products of $\Delta 6$ -desaturase.

Figures 9 and 10 depict GC profiles of the
FAMES derived from leaf and seed tissue, respectively,
of control and transformed tobacco plants. Figure 9A
provides the profile of leaf tissue of wild-type
25 tobacco (xanthi); Figure 9B provides the profile of
leaf tissue from a tobacco plant transformed with the
borage Δ -6 desaturase under the transcriptional
control of the 35S CaMV promoter (pBI 121 Δ ⁶NOS).
Peaks correspond to 18:2, 18:3 γ (GLA), 18:3 α and 18:4
30 (octadecanonic acid). Figure 10A shows the GC profile
of seeds of a wild-type tobacco; Figure 10B shows the

1 profile of seed tissue of a tobacco plant transformed
with pBI 121 Δ^6 NOS. Peaks correspond to 18:2,
18:3 γ (GLA) and 18:3 α .

5 The relative distribution of the C₁₈ fatty
acids in control and transgenic tobacco seeds is shown
in Table 4.

TABLE 4

	Fatty Acid	Xanthi	pBI121 Δ^6 NOS
10	18:0	4.0%	2.5%
	18:1	13%	13%
	18:2	82%	82%
	18:3 γ (GLA)	-	2.7%
15	18:3 α	0.82%	1.4%

The foregoing results demonstrate that GLA
is incorporated into the triacylglycerides of
transgenic tobacco leaves and seeds containing the
20 borage Δ^6 -desaturase.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Rhone-Poulenc Agrochimie
- (ii) TITLE OF INVENTION: PRODUCTION OF GAMMA LINOLENIC ACID BY A
DELTA 6-DESATURASE
- (iii) NUMBER OF SEQUENCES: 25
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Scully, Scott, Murphy & Presser
 - (B) STREET: 400 Garden City Plaza
 - (C) CITY: Garden City
 - (D) STATE: New York
 - (E) COUNTRY: United States
 - (F) ZIP: 11530
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE: 30-DEC-1994
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Presser, Leopold
 - (B) REGISTRATION NUMBER: 19,827
 - (C) REFERENCE/DOCKET NUMBER: 8383ZYXW
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (516) 742-4343
 - (B) TELEFAX: (516) 742-4366
 - (C) TELEX: 230 901 SANS UR

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 3588 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 2002..3081

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCTAGCCACC AGTGACGATG CCTTGAATTT GGCCATTCTG ACCCAGGCCC GTATTCTGAA	60
TCCCCGCATT CGCATTGTTA ATCGTTTGTT CAACCATGCC CTGGGTAAAC GTTTAGACAC	120
CACCTTGCCA GACCACGTTA GTTTGAGTGT TTCCGCCCTG GCGGCCCCGA TTTTTCCTT	180
TGCGGCTTTG GGCAATCAGG CGATCGGGCA ATTGCGTTT TTTGACCAGA CTTGGCCCAT	240
TCAGGAAATT GTCATTCACC AAGACCATCC CTGGCTCAAT TTACCCCTGG CGGATTTATG	300
GGATGATCCG AGCCGAATGT TGATCTATTA CCTACCGGCC CACAGTGAAA CGGATTTAGT	360
AGGCGCAGTG GTGAATAATT TAACGTTGCA ATCTGGGGAC CATTTAATAG TGGGACAAAA	420
ACCCCAACCC AAGACCAAAC GCGATCGCC TTGGCGCAA TTTCCAAAC TGATTACCAA	480
CCTGCGGGAG TATCAGCGGT ATGTCCAACA GGTGATATGG GTGGTGTGT TTTATTGTT	540
GATGATTTTT CTGGCCACCT TCATCTACGT TTCCATTGAT CAACATATTG CCCCAGTGGA	600
CGCGTTGTAT TTTTCCGTGG GCATGATTAC CGGGGCCGGT GGCAAGGAAG AGGTGGCCGA	660
AAAGTCCCC GATATCATCA AAGTATTCAC AGTGGTGATG ATGATCGCCG GGGCGGGGT	720
GATTGGTATT TGTTATGCCC TACTGAATGA TTTCATCCTT GGCAGTCGCT TTAGTCAGTT	780
TTTGGATGCG GCCAAGTTAC CCGATCGCCA TCACATCATC ATTTGTGGGC TGGGGGGAGT	840
GAGCATGGCC ATTATTGAAG AGTTAATTCA CCAGGGCCAT GAAATTGTGG TAATCGAAAA	900
GGATACAGAT AATCGTTTCT TGCATACGGC CCGCTCCCTG GGGGTGCCCC TAATTGTGGA	960
GGATGCCCCG CTAGAAAGAA CGTTGGCCTG CGCCAATATC AACCGAGCCG AAGCCATTGT	1020
GGTGGCCACC AGCGACGACA CCGTTAACTT GGAAATTGGC CTAAGTGGCA AGGCGATCGC	1080
CCCTAGCCTG CCAGTGGTGT TGCCTTGCCA GGATGCCAG TTTAGCCTGT CCCTGCAGGA	1140
AGTATTTGAA TTTGAAACGG TGCTTTGTCC GGCAGGAATTG GCCACCTATT CCTTTGCGGC	1200
GGCGGCCCTG GGGGGCAAAA TTTTGGGCAA CGGCATGACC GATGATTTCG TGTGGGTAGC	1260
CCTAGCCACC TTAATCACTC CTAACCATCC CTTTGCCGAC CAATTGGTTA AAATTGCAGC	1320
CCAAAAGTCT GATTTTCGTT CCCTCTATCT AGAACGGGGT GGCAAAACCA TCCATAGCTG	1380
GGAATTATTG GGTACCCATC TCGACTCTGG AGACGTGTTG TATTTAACCA TGCCCGCCAC	1440
TGCCCTAGAG CAACTTTGGC GATCGCCCCG TGCCACTGCT GATCCTCTGG ACTCTTTTTT	1500

GGTTTAGCAT GGGGGGATGG AACTCTTGAC TCGGCCCAAT GGTGATCAAG AAAGAACGCT	1560
TTGTCTATGT TTAGTATTTT TAAGTTAACC AACAGCAGAG GATAACTTCC AAAAGAAATT	1620
AAGCTCAAAA AGTAGCAAAA TAAGTTTAAT TCATAACTGA GTTTTACTGC TAAACAGCGG	1680
TGCAAAAAAG TCAGATAAAA TAAAAGCTTC ACTTCGGTTT TATATTGTGA CCATGGTTCC	1740
CAGGCATCTG CTCTAGGGAG TTTTCCGCT GCCTTTAGAG AGTATTTTCT CCAAGTCGGC	1800
TAACTCCCCC ATTTTtaggc AAAATCATAT ACAGACTATC CCAATATTGC CAGAGCTTTG	1860
ATGACTCACT GTAGAAGGCA GACTAAAATT CTAGCAATGG ACTCCCAGTT GGAATAAATT	1920
TTTAGTCTCC CCCGGCGCTG GAGTTTTTTT GTAGTTAATG GCGGTATAAT GTGAAAGTTT	1980
TTTATCTATT TAAATTTATA A ATG CTA ACA GCG GAA AGA ATT AAA TTT ACC	2031
Met Leu Thr Ala Glu Arg Ile Lys Phe Thr	10
1 5	
CAG AAA CGG GGG TTT CGT CGG GTA CTA AAC CAA CGG GTG GAT GCC TAC	2079
Gln Lys Arg Gly Phe Arg Arg Val Leu Asn Gln Arg Val Asp Ala Tyr	25
15 20	
TTT GCC GAG CAT GGC CTG ACC CAA AGG GAT AAT CCC TCC ATG TAT CTG	2127
Phe Ala Glu His Gly Leu Thr Gln Arg Asp Asn Pro Ser Met Tyr Leu	40
30 35	
AAA ACC CTG ATT ATT GTG CTC TGG TTG TTT TCC GCT TGG GCC TTT GTG	2175
Lys Thr Leu Ile Ile Val Leu Trp Leu Phe Ser Ala Trp Ala Phe Val	55
45 50	
CTT TTT GCT CCA GTT ATT TTT CCG GTG CGC CTA CTG GGT TGT ATG GTT	2223
Leu Phe Ala Pro Val Ile Phe Pro Val Arg Leu Leu Gly Cys Met Val	70
60 65	
TTG GCG ATC GCC TTG GCG GCC TTT TCC TTC AAT GTC GGC CAC GAT GCC	2271
Leu Ala Ile Ala Leu Ala Ala Phe Ser Phe Asn Val Gly His Asp Ala	90
75 80	
AAC CAC AAT GCC TAT TCC TCC AAT CCC CAC ATC AAC CGG GTT CTG GGC	2319
Asn His Asn Ala Tyr Ser Ser Asn Pro His Ile Asn Arg Val Leu Gly	105
95 100	
ATG ACC TAC GAT TTT GTC GGG TTA TCT AGT TTT CTT TGG CGC TAT CGC	2367
Met Thr Tyr Asp Phe Val Gly Leu Ser Ser Phe Leu Trp Arg Tyr Arg	120
110 115	
CAC AAC TAT TTG CAC CAC ACC TAC ACC AAT ATT CTT GGC CAT GAC GTG	2415
His Asn Tyr Leu His His Thr Tyr Thr Asn Ile Leu Gly His Asp Val	135
125 130	
GAA ATC CAT GGA GAT GGC GCA GTA CGT ATG AGT CCT GAA CAA GAA CAT	2463
Glu Ile His Gly Asp Gly Ala Val Arg Met Ser Pro Glu Gln Glu His	150
140 145	

TTTGAGGGGG TTCATTGGCC GCAGTTTCAA GCTGACCTAG GAGGCAAAGA TTGGGTGATT 3268
 TTGCTCAAAT CCGCTGGGAT ATTGAAAGGC TTCACCACCT TTGGTTTCTA CCCTGCTCAA 3328
 TGGGAAGGAC AAACCGTCAG AATTGTTTAT TCTGGTGACA CCATCACCGA CCCATCCATG 3388
 TGGTCTAACC CAGCCCTGGC CAAGGCTTGG ACCAAGGCCA TGCAAATTCT CCACGAGGCT 3448
 AGGCCAGAAA AATTATATTG GCTCCTGATT TCTTCCGGCT ATCGCACCTA CCGATTTTGT 3508
 AGCATTTTTG CCAAGGAATT CTATCCCCAC TATCTCCATC CCACTCCCCC GCCTGTACAA 3568
 AATTTTATCC ATCAGCTAGC 3588

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 359 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Leu Thr Ala Glu Arg Ile Lys Phe Thr Gln Lys Arg Gly Phe Arg
 1 5 10 15
 Arg Val Leu Asn Gln Arg Val Asp Ala Tyr Phe Ala Glu His Gly Leu
 20 25 30
 Thr Gln Arg Asp Asn Pro Ser Met Tyr Leu Lys Thr Leu Ile Ile Val
 35 40 45
 Leu Trp Leu Phe Ser Ala Trp Ala Phe Val Leu Phe Ala Pro Val Ile
 50 55 60
 Phe Pro Val Arg Leu Leu Gly Cys Met Val Leu Ala Ile Ala Leu Ala
 65 70 75 80
 Ala Phe Ser Phe Asn Val Gly His Asp Ala Asn His Asn Ala Tyr Ser
 85 90 95
 Ser Asn Pro His Ile Asn Arg Val Leu Gly Met Thr Tyr Asp Phe Val
 100 105 110
 Gly Leu Ser Ser Phe Leu Trp Arg Tyr Arg His Asn Tyr Leu His His
 115 120 125
 Thr Tyr Thr Asn Ile Leu Gly His Asp Val Glu Ile His Gly Asp Gly
 130 135 140
 Ala Val Arg Met Ser Pro Glu Gln Glu His Val Gly Ile Tyr Arg Phe
 145 150 155 160

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Gln Gln Phe Tyr Ile Trp Gly Leu Tyr Leu Phe Ile Pro Phe Tyr Trp
 165 170 175
 Phe Leu Tyr Asp Val Tyr Leu Val Leu Asn Lys Gly Lys Tyr His Asp
 180 185 190
 His Lys Ile Pro Pro Phe Gln Pro Leu Glu Leu Ala Ser Leu Leu Gly
 195 200 205
 Ile Lys Leu Leu Trp Leu Gly Tyr Val Phe Gly Leu Pro Leu Ala Leu
 210 215 220
 Gly Phe Ser Ile Pro Glu Val Leu Ile Gly Ala Ser Val Thr Tyr Met
 225 230 235 240
 Thr Tyr Gly Ile Val Val Cys Thr Ile Phe Met Leu Ala His Val Leu
 245 250 255
 Glu Ser Thr Glu Phe Leu Thr Pro Asp Gly Glu Ser Gly Ala Ile Asp
 260 265 270
 Asp Glu Trp Ala Ile Cys Gln Ile Arg Thr Thr Ala Asn Phe Ala Thr
 275 280 285
 Asn Asn Pro Phe Trp Asn Trp Phe Cys Gly Gly Leu Asn His Gln Val
 290 295 300
 Thr His His Leu Phe Pro Asn Ile Cys His Ile His Tyr Pro Gln Leu
 305 310 315 320
 Glu Asn Ile Ile Lys Asp Val Cys Gln Glu Phe Gly Val Glu Tyr Lys
 325 330 335
 Val Tyr Pro Thr Phe Lys Ala Ala Ile Ala Ser Asn Tyr Arg Trp Leu
 340 345 350
 Glu Ala Met Gly Lys Ala Ser
 355

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1884 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AGCTTCACTT CGGTTTTATA TTGTGACCAT GGTTCCCAGG CATCTGCTCT AGGGAGTTTT 60
 TCCGCTGCCT TTAGAGAGTA TTTTCTCCAA GTCGGCTAAC TCCCCCATTT TTAGGCAAAA 120

TCATATACAG	ACTATCCCAA	TATTGCCAGA	GCTTTGATGA	CTCACTGTAG	AAGGCAGACT	180
AAAATTCTAG	CAATGGACTC	CCAGTTGGAA	TAAATTTTFA	GTCTCCCCCG	GCGCTGGAGT	240
TTTTTTGTAG	TTAATGGCGG	TATAATGTGA	AAGTTTTTTA	TCTATTTAAA	TTTATAAATG	300
CTAACAGCGG	AAAGAATTAA	ATTTACCCAG	AAACGGGGGT	TTCGTCGGGT	ACTAAACCAA	360
CGGGTGGATG	CCTACTTTGC	CGAGCATGGC	CTGACCCAAA	GGGATAATCC	CTCCATGTAT	420
CTGAAAACCC	TGATTATTGT	GCTCTGGTTG	TTTCCGCTT	GGGCCTTTGT	GCTTTTTGCT	480
CCAGTTATTT	TTCCGGTGCG	CCTACTGGGT	TGTATGGTTT	TGGCGATCGC	CTTGGCGGCC	540
TTTTCCTTCA	ATGTCGGCCA	CGATGCCAAC	CACAATGCCT	ATTCCTCCAA	TCCCCACATC	600
AACCGGGTTC	TGGGCATGAC	CTACGATTTT	GTCGGGTAT	CTAGTTTTCT	TTGGCGCTAT	660
CGCCACAAC	ATTTGCACCA	CACCTACACC	AATATTCTTG	GCCATGACGT	GGAAATCCAT	720
GGAGATGGCG	CAGTACGTAT	GAGTCCTGAA	CAAGAACATG	TTGGTATTTA	TCGTTTCCAG	780
CAATTTTATA	TTTGGGGTTT	ATATCTTTTC	ATTCCCTTTT	ATTGGTTTCT	CTACGATGTC	840
TACCTAGTGC	TTAATAAAGG	CAAATATCAC	GACCATAAAA	TTCCTCCTTT	CCAGCCCCCTA	900
GAATTAGCTA	GTTTGCTAGG	GATTAAGCTA	TTATGGCTCG	GCTACGTTTT	CGGCTTACCT	960
CTGGCTCTGG	GCTTTTCCAT	TCCTGAAGTA	TTAATTGGTG	CTTCGGTAAC	CTATATGACC	1020
TATGGCATCG	TGGTTTGCAC	CATCTTTATG	CTGGCCCATG	TGTTGGAATC	AACTGAATTT	1080
CTCACCCCG	ATGGTGAATC	CGGTGCCATT	GATGACGAGT	GGGCTATTTG	CCAAATTCGT	1140
ACCACGGCCA	ATTTTGCCAC	CAATAATCCC	TTTTGGAACT	GGTTTTGTGG	CGGTTTAAAT	1200
CACCAAGTTA	CCCACCATCT	TTTCCCCAAT	ATTTGTCATA	TCACTATCC	CCAATTGGAA	1260
AATATTATTA	AGGATGTTTG	CCAAGAGTTT	GGTGTGGAAT	ATAAAGTTTA	TCCCACCTTC	1320
AAAGCGGCGA	TCGCCTCTAA	CTATCGCTGG	CTAGAGGCCA	TGGGCAAAGC	ATCGTGACAT	1380
TGCCTTGGA	TTGAAGCAAA	ATGGCAAAAT	CCCTCGTAAA	TCTATGATCG	AAGCCTTTCT	1440
GTTGCCCGCC	GACCAAATCC	CCGATGCTGA	CCAAAGGTTG	ATGTTGGCAT	TGCTCCAAAC	1500
CCACTTTGAG	GGGGTTCATT	GGCCGCAGTT	TCAAGCTGAC	CTAGGAGGCA	AAGATTGGGT	1560
GATTTTGCTC	AAATCCGCTG	GGATATTGAA	AGGCTTCACC	ACCTTTGGTT	TCTACCCTGC	1620
TCAATGGGAA	GGACAAACCG	TCAGAATTGT	TTATTCTGGT	GACACCATCA	CCGACCCATC	1680
CATGTGGTCT	AACCCAGCCC	TGGCCAAGGC	TTGGACCAAG	GCCATGCAAA	TTCTCCACGA	1740
GGCTAGGCCA	GAAAAATTAT	ATTGGCTCCT	GATTTCTTCC	GGCTATCGCA	CCTACCGATT	1800

TTTGAGCATT TTTGCCAAGG AATTCTATCC CCACTATCTC CATCCCACTC CCCC GCCTGT 1860
 ACAAAATTTT ATCCATCAGC TAGC 1884

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1685 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AATATCTGCC TACCCTCCCA AAGAGAGTAG TCATTTTTC TCAATGGCTG CTCAAATCAA 60
 GAAATACATT ACCTCAGATG AACTCAAGAA CCACGATAAA CCCGGAGATC TATGGATCTC 120
 GATTCAAGGG AAAGCCTATG ATGTTTCGGA TTGGGTGAAA GACCATCCAG GTGGCAGCTT 180
 TCCCTTGAAG AGTCTTGCTG GTCAAGAGGT AACTGATGCA TTTGTTGCAT TCCATCCTGC 240
 CTCTACATGG AAGAATCTTG ATAAGTTTTT CACTGGGTAT TATCTTAAAG ATTACTCTGT 300
 TTCTGAGGTT TCTAAAGATT ATAGGAAGCT TGTGTTTGAG TTTTCTAAAA TGGGTTTGTA 360
 TGACAAAAAA GGTCATATTA TGTTTGCAAC TTTGTGCTTT ATAGCAATGC TGTTTGCTAT 420
 GAGTGTTTAT GGGGTTTTGT TTTGTGAGGG TGTTTTGGTA CATTTGTTTT CTGGGTGTTT 480
 GATGGGGTTT CTTTGGATTG AGAGTGGTTG GATTGGACAT GATGCTGGGC ATTATATGGT 540
 AGTGTCTGAT TCAAGGCTTA ATAAGTTTAT GGGTATTTTT GCTGCAAATT GTCTTTCAGG 600
 AATAAGTATT GGTTGGTGGA AATGGAACCA TAATGCACAT CACATTGCCT GTAATAGCCT 660
 TGAATATGAC CCTGATTTAC AATATATACC ATTCCTTGTT GTGTCTTCCA AGTTTTTTGG 720
 TTCCTCACC TCTCATTCT ATGAGAAAAG GTTGACTTTT GACTCTTTAT CAAGATTCTT 780
 TGTAAGTTAT CAACATTGGA CATTTTACCC TATTATGTGT GCTGCTAGGC TCAATATGTA 840
 TGTACAATCT CTCATAATGT TGTTGACCAA GAGAAATGTG TCCTATCGAG CTCAGGAACT 900
 CTTGGGATGC CTAGTGTCT CGATTTGGTA CCCGTTGCTT GTTTCTTGTT TGCCTAATTG 960
 GGGTGAAAGA ATTATGTTTG TTATTGCAAG TTTATCAGTG ACTGGAATGC AACAAGTTCA 1020
 GTTCTCCTTG AACCATTCT CTTCAAGTGT TTATGTTGGA AAGCCTAAAG GGAATAATTG 1080
 GTTTGAGAAA CAAACGGATG GGACACTTGA CATTTCTTGT CCTCCTTGGA TGGATTGGTT 1140
 TCATGGTGGA TTGCAATTCC AAATTGAGCA TCATTTGTTT CCCAAGATGC CTAGATGCAA 1200

CCTTAGGAAA ATCTCGCCCT ACGTGATCGA GTTATGCAAG AACATAATT TGCCTTACAA 1260
 TTATGCATCT TTCTCCAAGG CCAATGAAAT GACACTCAGA ACATTGAGGA ACACAGCATT 1320
 GCAGGCTAGG GATATAACCA AGCCGCTCCC GAAGAATTTG GTATGGGAAG CTCTTCACAC 1380
 TCATGGTTAA AATTACCCTT AGTTCATGTA ATAATTTGAG ATTATGTATC TCCTATGTTT 1440
 GTGTCTTGTC TTGGTTCTAC TTGTTGGAGT CATTGCAACT TGTCTTTTAT GGTTTATTAG 1500
 ATGTTTTTTA ATATATTTTA GAGGTTTTGC TTTCATCTCC ATTATTGATG AATAAGGAGT 1560
 TGCATATTGT CAATTGTTGT GCTCAATATC TGATATTTTG GAATGTACTT TGTACCACTG 1620
 TGTTTTCAGT TGAAGCTCAT GTGTACTTCT ATAGACTTTG TTTAAATGGT TATGTCATGT 1680
 TATTT 1685

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 448 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met	Ala	Ala	Gln	Ile	Lys	Lys	Tyr	Ile	Thr	Ser	Asp	Glu	Leu	Lys	Asn
1			5					10					15		
His	Asp	Lys	Pro	Gly	Asp	Leu	Trp	Ile	Ser	Ile	Gln	Gly	Lys	Ala	Tyr
		20					25						30		
Asp	Val	Ser	Asp	Trp	Val	Lys	Asp	His	Pro	Gly	Gly	Ser	Phe	Pro	Leu
		35				40						45			
Lys	Ser	Leu	Ala	Gly	Gln	Glu	Val	Thr	Asp	Ala	Phe	Val	Ala	Phe	His
		50				55					60				
Pro	Ala	Ser	Thr	Trp	Lys	Asn	Leu	Asp	Lys	Phe	Phe	Thr	Gly	Tyr	Tyr
65				70					75					80	
Leu	Lys	Asp	Tyr	Ser	Val	Ser	Glu	Val	Ser	Lys	Asp	Tyr	Arg	Lys	Leu
			85					90						95	
Val	Phe	Glu	Phe	Ser	Lys	Met	Gly	Leu	Tyr	Asp	Lys	Lys	Gly	His	Ile
			100					105					110		
Met	Phe	Ala	Thr	Leu	Cys	Phe	Ile	Ala	Met	Leu	Phe	Ala	Met	Ser	Val
		115					120					125			
Tyr	Gly	Val	Leu	Phe	Cys	Glu	Gly	Val	Leu	Val	His	Leu	Phe	Ser	Gly
		130				135					140				

Cys Leu Met Gly Phe Leu Trp Ile Gln Ser Gly Trp Ile Gly His Asp
 145 150 155 160
 Ala Gly His Tyr Met Val Val Ser Asp Ser Arg Leu Asn Lys Phe Met
 165 170 175
 Gly Ile Phe Ala Ala Asn Cys Leu Ser Gly Ile Ser Ile Gly Trp Trp
 180 185 190
 Lys Trp Asn His Asn Ala His His Ile Ala Cys Asn Ser Leu Glu Tyr
 195 200 205
 Asp Pro Asp Leu Gln Tyr Ile Pro Phe Leu Val Val Ser Ser Lys Phe
 210 215 220
 Phe Gly Ser Leu Thr Ser His Phe Tyr Glu Lys Arg Leu Thr Phe Asp
 225 230 235 240
 Ser Leu Ser Arg Phe Phe Val Ser Tyr Gln His Trp Thr Phe Tyr Pro
 245 250 255
 Ile Met Cys Ala Ala Arg Leu Asn Met Tyr Val Gln Ser Leu Ile Met
 260 265 270
 Leu Leu Thr Lys Arg Asn Val Ser Tyr Arg Ala Gln Glu Leu Leu Gly
 275 280 285
 Cys Leu Val Phe Ser Ile Trp Tyr Pro Leu Leu Val Ser Cys Leu Pro
 290 295 300
 Asn Trp Gly Glu Arg Ile Met Phe Val Ile Ala Ser Leu Ser Val Thr
 305 310 315 320
 Gly Met Gln Gln Val Gln Phe Ser Leu Asn His Phe Ser Ser Ser Val
 325 330 335
 Tyr Val Gly Lys Pro Lys Gly Asn Asn Trp Phe Glu Lys Gln Thr Asp
 340 345 350
 Gly Thr Leu Asp Ile Ser Cys Pro Pro Trp Met Asp Trp Phe His Gly
 355 360 365
 Gly Ser Gln Phe Gln Ile Glu His His Leu Phe Pro Lys Met Pro Arg
 370 375 380
 Cys Asn Leu Arg Lys Ile Ser Pro Tyr Val Ile Glu Leu Cys Lys Lys
 385 390 395 400
 His Asn Leu Pro Tyr Asn Tyr Ala Ser Phe Ser Lys Ala Asn Glu Met
 405 410 415
 Thr Leu Arg Thr Leu Arg Asn Thr Ala Leu Gln Ala Arg Asp Ile Thr
 420 425 430
 Lys Pro Leu Pro Lys Asn Leu Val Trp Glu Ala Leu His Thr His Gly
 435 440 445

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Trp Ile Gly His Asp Ala Gly His
1 5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Asn Val Gly His Asp Ala Asn His
1 5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Val Leu Gly His Asp Cys Gly His
1 5

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

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Val Ile Ala His Glu Cys Gly His
1 5

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Val Ile Gly His Asp Cys Ala His
1 5

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Val Val Gly His Asp Cys Gly His
1 5

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

His Asn Ala His His
1 5

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

His Asn Tyr Leu His His
1 5

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

His Arg Thr His His
1 5

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

His Arg Arg His His
1 5

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

His Asp Arg His His
1 5

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

His Asp Gln His His
1 5

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

His Asp His His His
1 5

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

His Asn His His His
1 5

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Phe Gln Ile Glu His His
1 5

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

His Gln Val Thr His His
1 5

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

His Val Ile His His
1 5

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid

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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

His Val Ala His His
1 5

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

His Ile Pro His His
1 5

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

His Val Pro His His
1 5

1 WHAT IS CLAIMED:

1. An isolated nucleic acid encoding a borage $\Delta 6$ -desaturase.

5

2. The isolated nucleic acid of Claim 1 comprising the nucleotide sequence of SEQ ID NO: 4.

10 3. An isolated nucleic acid that codes for the amino acid sequence of SEQ ID NO: 5.

4. A vector comprising the nucleic acid of any one Claims 1-3.

15

5. An expression vector comprising the isolated nucleic acid of any one of Claims 1-3 operably linked to a promoter and optionally a termination signal capable of effecting expression of the gene product of said isolated nucleic acid.

20

6. The expression vector of Claim 5 wherein said promoter is a Δ -6 desaturase promoter, an Anabaena carboxylase promoter, a helianthinin promoter, a glycinin promoter, a napin promoter, the 35S promoter from CaMV, or
25 a helianthinin tissue-specific promoter.

7. The expression vector of Claim 5 wherein said promoter is constitutive or tissue-specific.

30

8. The expression vector of Claim 5 wherein said termination signal is a Synechocystis termination

35

1 signal, a nopaline synthase termination signal, or a seed
termination signal.

5 9. A cell comprising the vector of any one of
Claims 4-8.

10 10. The cell of Claim 9 wherein said cell is an
animal cell, a bacterial cell, a plant cell or a fungal
cell.

11. A transgenic organism comprising the
isolated nucleic acid of any one of Claims 1-3.

15 12. A transgenic organism comprising the vector
of any one of Claims 4-8.

13. The transgenic organism of Claim 11 or 12
wherein said organism is a bacterium, a fungus, a plant or
an animal.

20 14. A plant or progeny of said plant which has
been regenerated from the plant cell of Claim 10.

25 15. The plant of Claim 14 wherein said plant is
a sunflower, soybean, maize, tobacco, peanut, carrot or
oil seed rape plant.

30 16. A method of producing a plant with
increased gamma linolenic acid (GLA) content which
comprises:

1 (a) transforming a plant cell with the isolated
nucleic acid of any one of Claims 1-3; and

(b) regenerating a plant with increased GLA
content from said plant cell.

5

17. A method of producing a plant with
increased gamma linolenic acid (GLA) content which
comprises:

(a) transforming a plant cell with the vector of
10 any one of Claims 4-8; and

(b) regenerating a plant with increased GLA
content from said plant cell.

18. The method of Claim 16 or 17 wherein said
15 plant is a sunflower, soybean, maize, tobacco, peanut,
carrot or oil seed rape plant.

19. A method of inducing production of gamma
linolenic acid (GLA) in an organism deficient or lacking
20 in GLA which comprises transforming said organism with the
isolated nucleic acid of any one of Claims 1-3.

20. A method of inducing production of gamma
linolenic acid (GLA) in an organism deficient or lacking
25 in GLA which comprises transforming said organism with the
vector of any one of Claims 4-8.

21. A method of inducing production of gamma
linolenic acid (GLA) in an organism deficient or lacking
30 in GLA and linoleic acid (LA) which comprises transforming
said organism with an isolated nucleic acid encoding

35

1 borage Δ 6-desaturase and an isolated nucleic acid encoding
12 Δ 12-desaturase.

22. The method of Claim 21 wherein said
5 isolated nucleic acid encoding Δ 6-desaturase comprises
nucleotides 44 to 1390 of SEQ. ID NO: 4.

23. A method of inducing production of
octadecatetraeonic acid in an organism deficient or
10 lacking in gamma linolenic acid which comprises
transforming said organism with the isolated nucleic acid
of any one of Claims 1-3.

24. A method of inducing production of
15 octadecatetraeonic acid in an organism deficient or
lacking in gamma linolenic acid which comprises
transforming said organism with the vector of any one of
Claims 4-8.

20 25. The method of Claim 23 or 24 wherein said
organism is a bacterium, a fungus, a plant or an animal.

26. A method of producing a plant with improved
chilling resistance which comprises:
25 (a) transforming a plant cell with the isolated
nucleic acid of any one of Claims 1-3; and
(b) regenerating said plant with improved
chilling resistance from said transformed plant cell.

27. A method of producing a plant with improved
30 chilling resistance which comprises:

1 (a) transforming a plant cell with the vector of
any one of Claims 4-8; and

(b) regenerating said plant with improved
chilling resistance from said transformed plant cell.

5

28. The method of Claim 26 or 27 wherein said
plant is a sunflower, soybean, maize, tobacco, peanut,
carrot or oil seed rape plant.

10

15

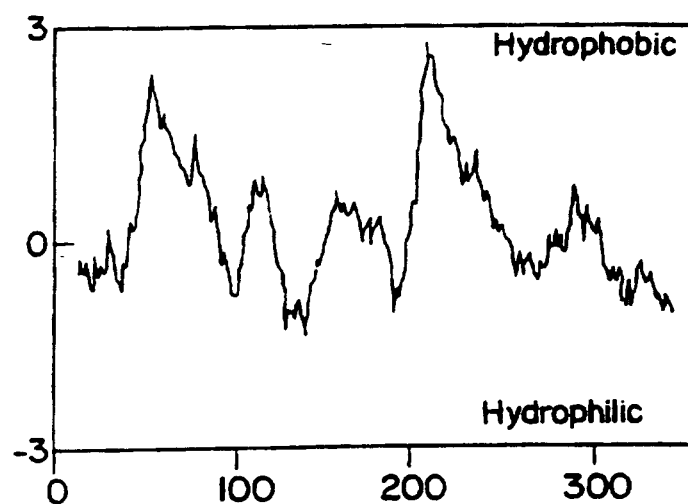
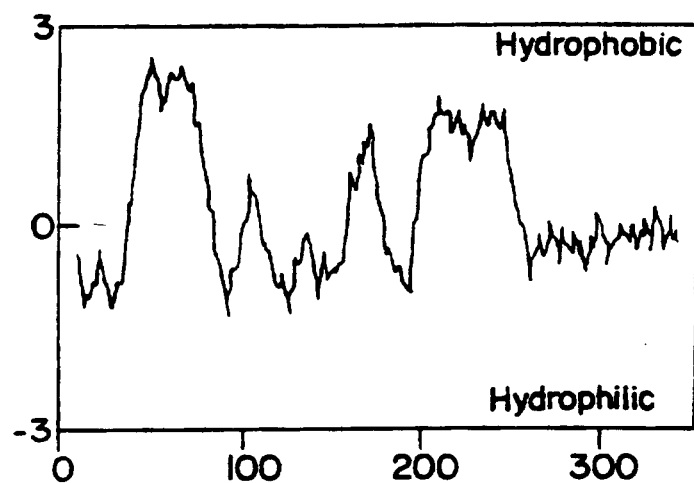
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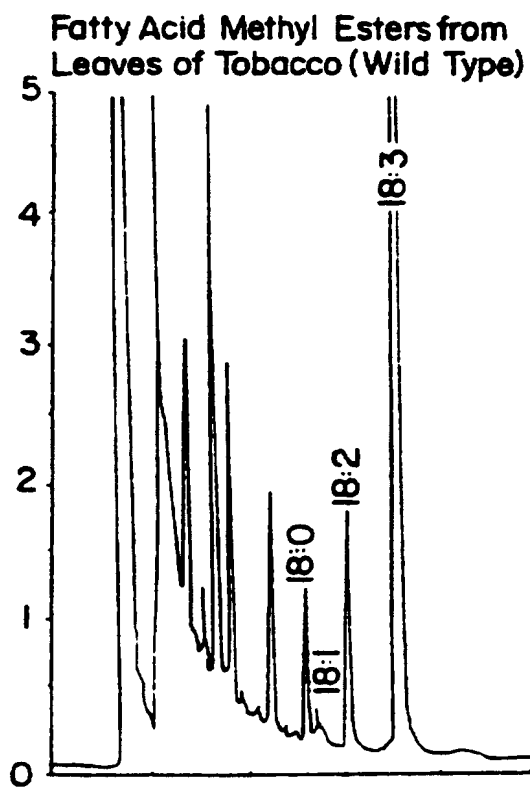


FIG. 4A

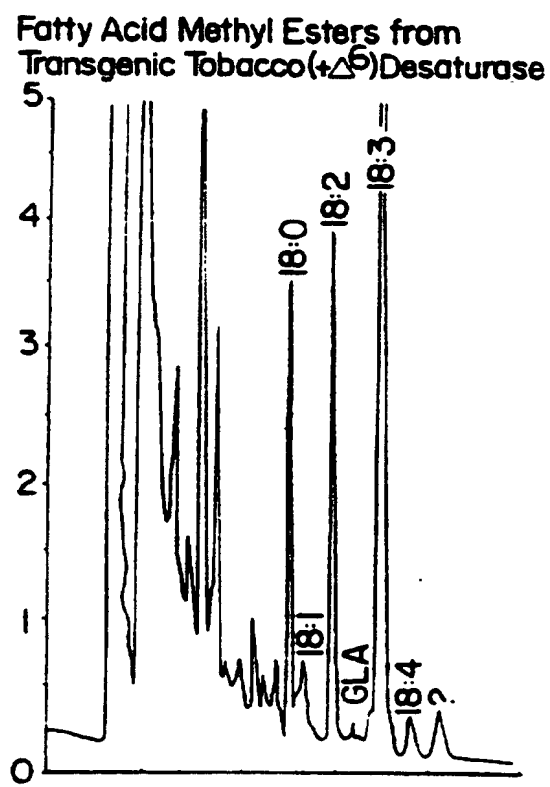


FIG. 4B

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FIG.5A

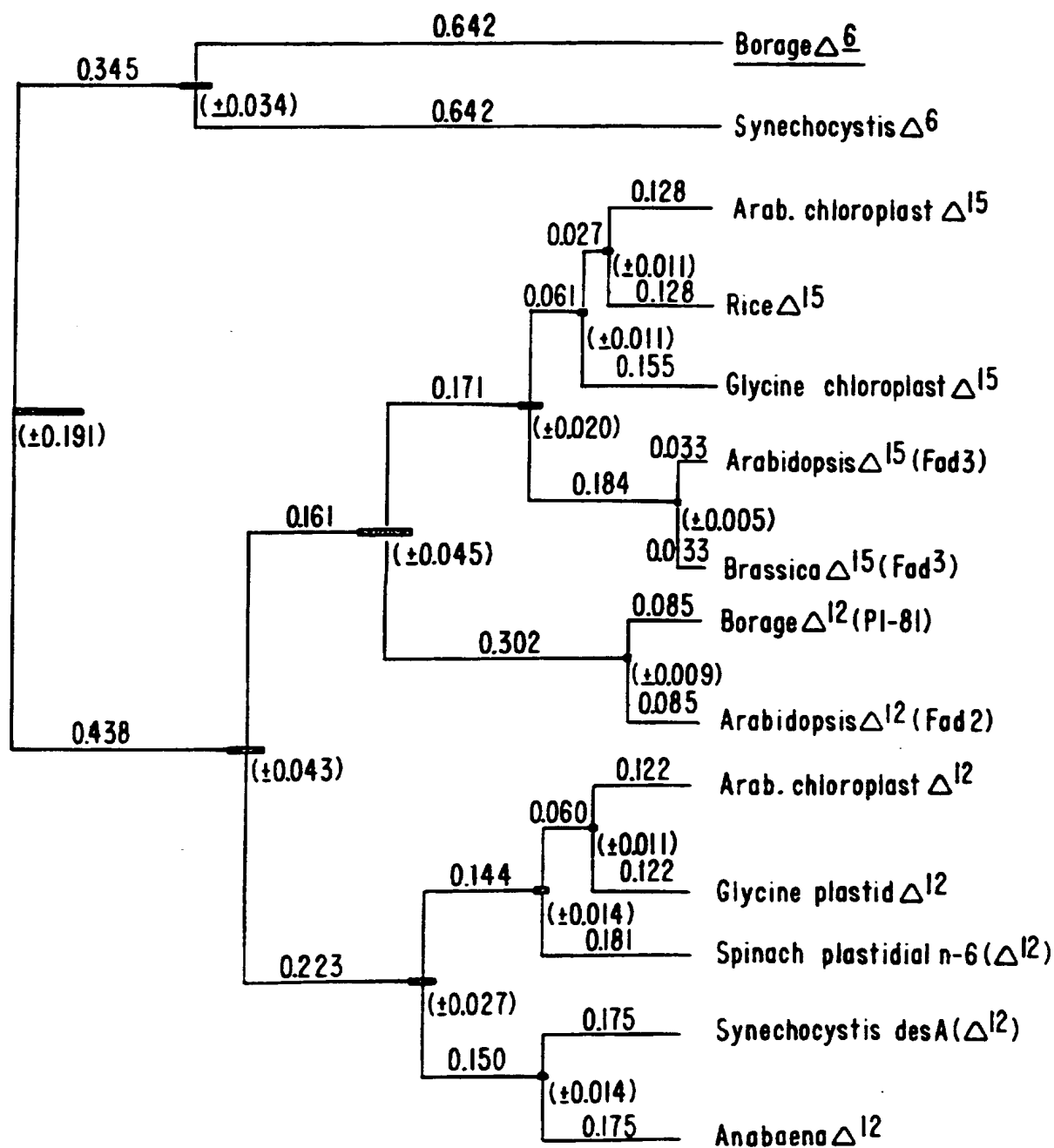
1 aatatctgcc taccctccca aagagagtag tcatcttttca tcaatggctg ctcaaatcaa gaaatacatt acctcagatg 80
 81 aactcaagaa ccacgataaa cccggagatc tatggatctc gattcaagg aaagcctatg atgtttcgga ttgggtgaaa 160
 161 gaccatccag gtggcagctt tcccttgaag agtcttgctg gtcaagaggt aactgatgca ttgttgcat tccatcctgc 240
 241 ctctacatgg aagaatcttg ataatgtttt cactgggtat tatcttaag attactctgt ttctgaggtt tctaaagatt 320
 321 ataggaagct tgtgtttgag ttttctaaaa tgggttttga tgacaaaaaa ggtcataatta tgtttgcaac ttgtgcttt 400
 401 atagcaatgc tgtttgctat gagtgtttat ggggttttgt ttgtgaggg tgttttgga catttgttt ctgggtgctt 480
 481 gatgggttt ctttggattc agagtggttg gattggacat gatgctgggc attatatggt agtctctgat tcaaggctta 560
 561 ataatgttat ggggtatttt gctgcaaat gtctttcagg aataagtatt ggttgggtga aatggaacca taatgcacat 640
 641 cacattgctt gtaatagcct tgaatatgac cctgatttac aatatatacc attccttgtt gtgtcttcca agtttttgg 720
 721 ttcaactacc tctcatttct atgagaaaaa gttgactttt gactctttat caagattcct tgtgaagttat caacattgga 800
 801 cattttacc tattatgtgt gctgctaggc tcaatatgta tgtacaatct ctcataatgt tgttgaccaa gagaaatgtg 880
 881 tcctatcag ctccaggaaact cttgggatgc ctagtgttct cgatttgga cccgttgctt gtttcttgtt tgcctaattg 960
 961 ggtgaaaga attatgtttg ttattgcaag ttatcagtg actggaatgc aacaagttca gttctccttg aaccttct 1040
 1041 ctcaagtgt ttatgttga aagcctaaag ggaataattg gtttgagaaa caaacggatg ggacacttga catttcttgt 1120
 1121 cctccttga tggattggtt tcatggtgga ttgcaattcc aaattgagca tcatttgttt cccaagatgc ctagatgcaa 1200
 1201 ccttaggaaa atctcgccct acgtgatcga gttatgcaag aaacataatt tgcctacaa ttatgcatct ttctccaagg 1280
 1281 ccaatgaaat gacactcaga acattgagga acacagcatt gcaggctagg gataaacca agccgctccc gaagaatttg 1360
 1361 gtatgggaag ctcttcacac tcatggttaa aattaccctt agttcatgta ataatgtgag attatgtatc tctatgttt 1440
 1441 gtgtcttgc ttggttctac ttggtggagt cattgcaact tgtcttttat ggtttattag atgttttta atatattta 1520
 1521 gaggttttgc ttcatctcc attattgatg aataaggagt tgcataattgt caattgttgt gctcaatc tgatatattg 1600
 1601 gaatgtactt tgtaccactg tgttttctagt tgaagctcat gtgtacttct atagactttg tttaaatggt tatgtcatgt 1680
 1681 tattt 1685

FIG.5B

1 MAAQIKKYIT SDELKNHDKP GDLWISIQGK AYDVSDWVKD HPGGSFPLKS LAGQEVTD AF VAFHPASTWK NLDKFFTGYY 80
 81 LKDYSVSEVS KDYRKLVFEE SKMGLYDKKG HIMFATLCFI AMLFAMSVYG VLFCEGVLVH LFSGCLMGFL WIQSGWIGHD 160
 161 AGHYMVVSDS RLNKFMGIFA ANCLSGISIG WWKWNHNAHH IACNSLEYDP DLQYIPFLVV SSKFFGSLTS HFYEKRLTFD 240
 241 SLSRFFVSQY HWTFFPIMCA ARLNMVQSL IMLLTNRNVS YRAQELLGCL VFSIWYPLL V SCLPNWGERI MFVIASLSVT 320
 321 GMOQVQFSLN HFSSSVYVGK PKGNWFEKQ TDGTLDISCP FMDWFHGGGL QFOI~~EH~~HFLF KMPRCNLRKI SPYVIELCKK 400
 401 HNLPPNYASF SKANEMTLRT LRNTALQARD ITKPLPKNLV WEALHTHG 448

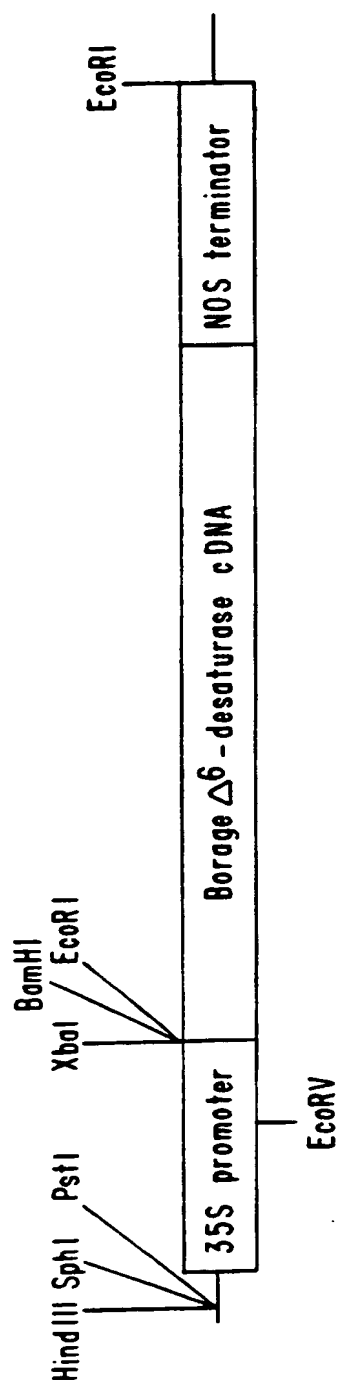
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FIG. 6



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FIG. 7



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FIG. 8A

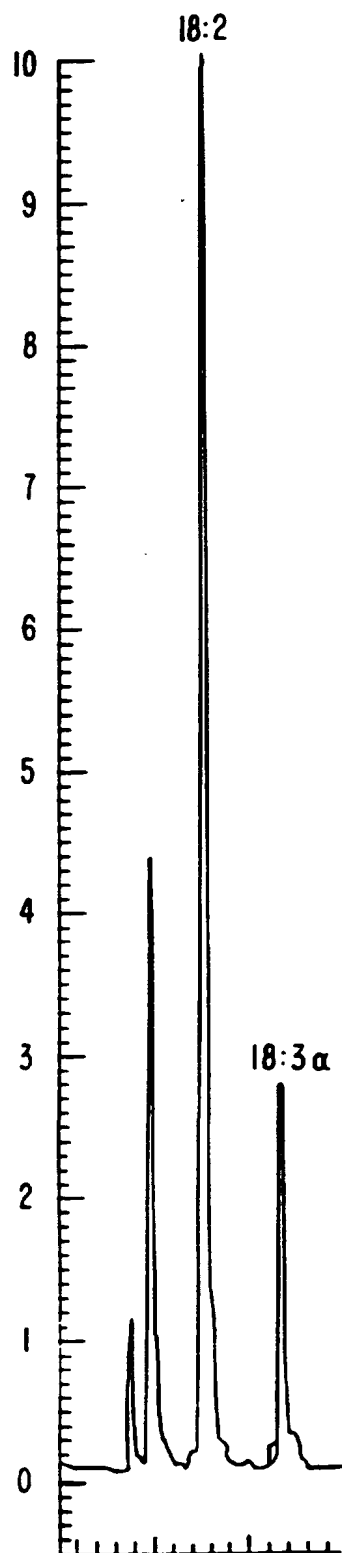


FIG. 8B

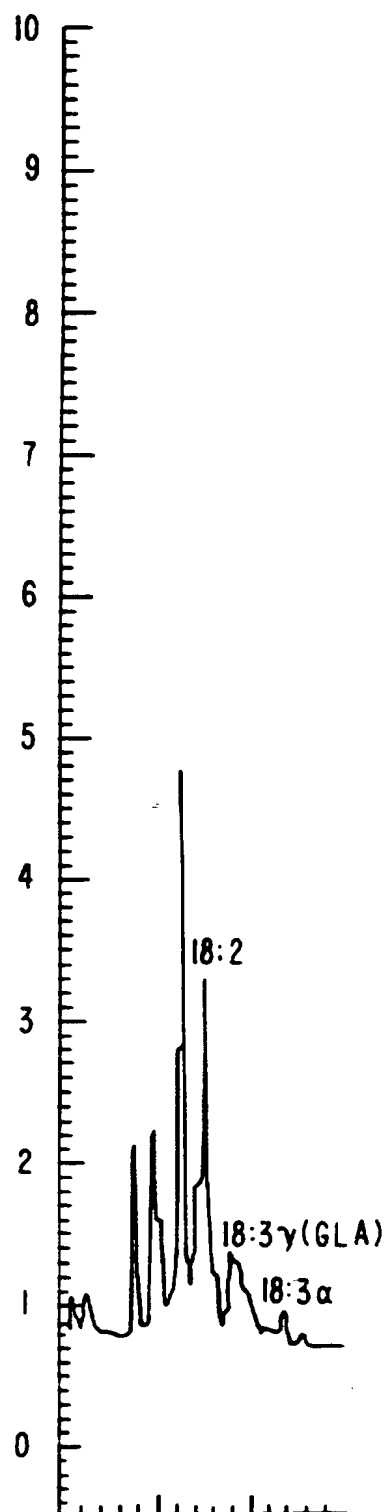
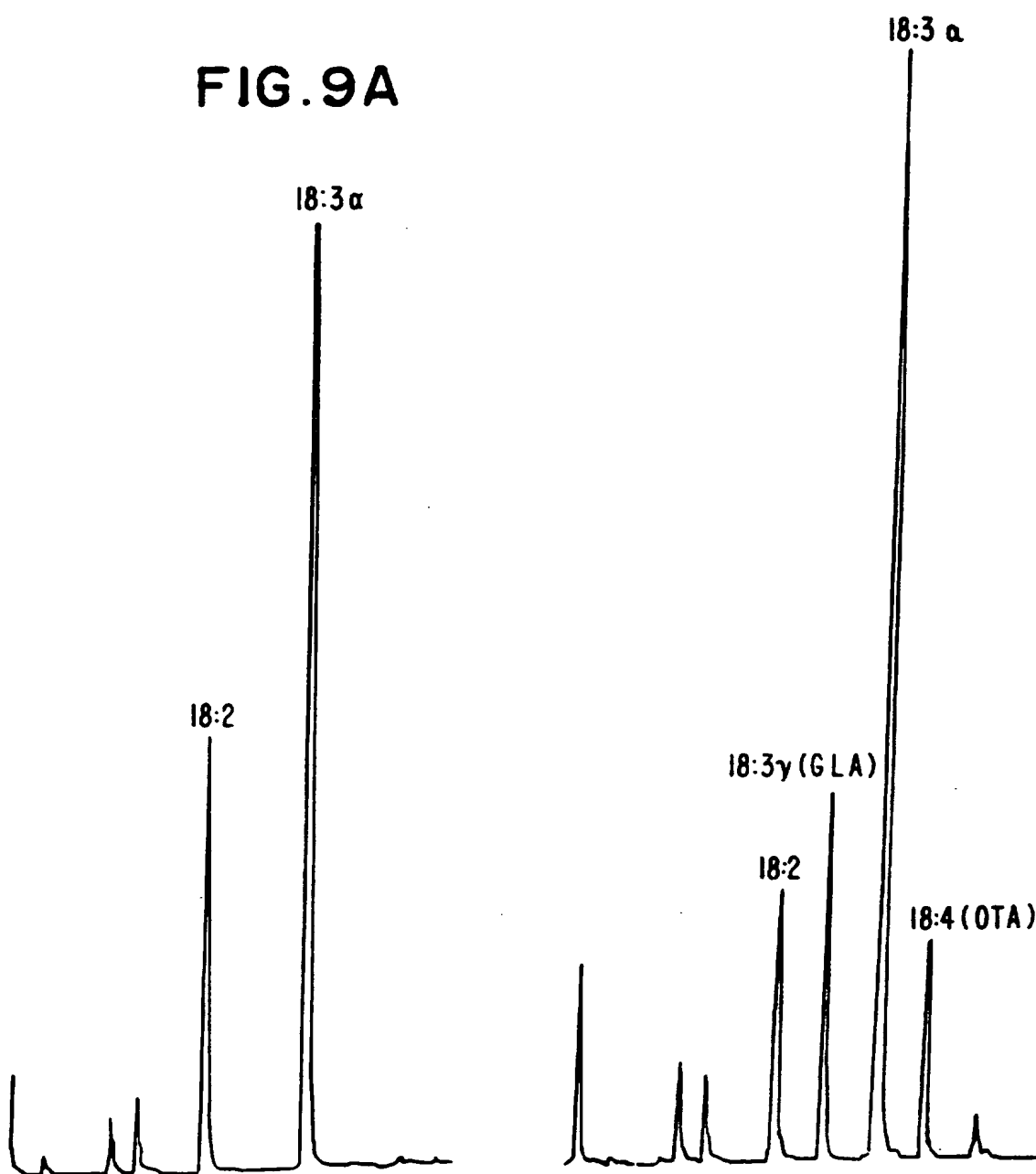


FIG. 9B



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FIG. 10A

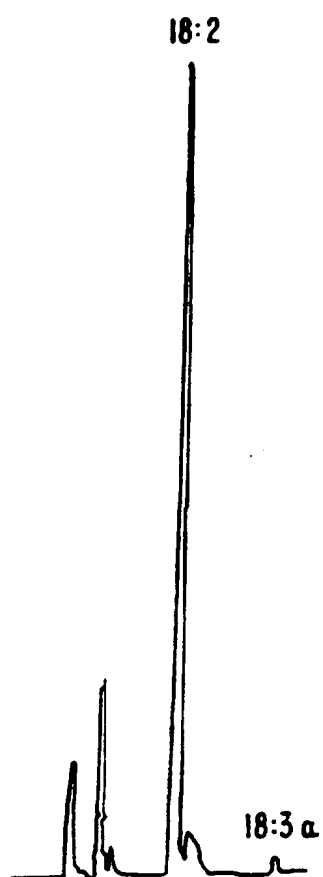
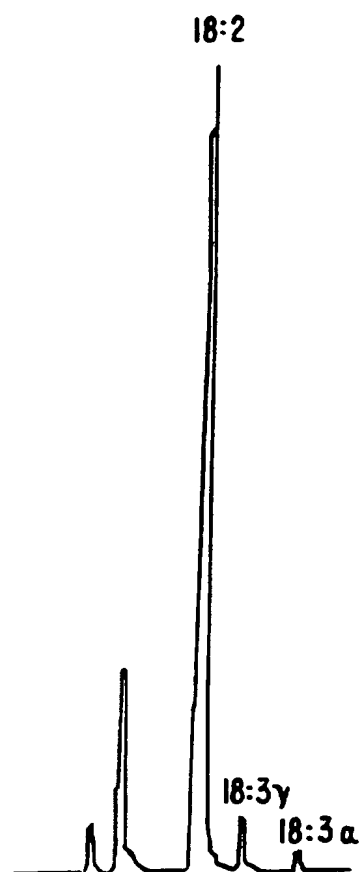


FIG. 10B





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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/53, 15/82, A01H 5/00	A3	(11) International Publication Number: WO 96/21022 (43) International Publication Date: 11 July 1996 (11.07.96)
(21) International Application Number: PCT/IB95/01167 (22) International Filing Date: 28 December 1995 (28.12.95) (30) Priority Data: 08/366,779 30 December 1994 (30.12.94) US (71) Applicant: RHONE-POULENC AGROCHIMIE [FR/FR]; 14-20, rue Pierre-Baizet, F-69263 Lyon (FR). (72) Inventors: THOMAS, Terry, L.; 3004 Normand, College Station, TX 77845 (US). REDDY, Avutu, S.; 3902 E. 29th Street #G11, Bryan, TX 77802 (US). NUCCIO, Michael; P.O. Box 553, College Station, TX 77841 (US). NUNBERG, Andrew, N.; 2804 B. Sprucewood Street, Bryan, TX 77801 (US). FREYSSINET, Georges, L.; 21, rue de Nervieux, F-69450 Saint-Cyr-au-Mont-d'Or (FR). (74) Agent: MITSCHERLICH & PARTNER; Sonnenstrasse 33, D-80331 München (DE).		(81) Designated States: AU, BR, CA, CN, JP, RO, RU, UA, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 12 September 1996 (12.09.96)

(54) Title: PRODUCTION OF GAMMA LINOLENIC ACID BY A $\Delta 6$ -DESATURASE**(57) Abstract**

Linoleic acid is converted into γ -linolenic acid by the enzyme $\Delta 6$ -desaturase. The present invention is directed to isolated nucleic acids comprising the $\Delta 6$ -desaturase gene. More particularly, the isolated nucleic acid comprises the promoter, coding region and termination regions of the $\Delta 6$ -desaturase gene. The present invention provides recombinant constructions comprising the $\Delta 6$ -desaturase coding region in functional combination with heterologous regulatory sequences. The nucleic acids and recombinant constructions of the instant invention are useful in the production of GLA in transgenic organisms.

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INTERNATIONAL SEARCH REPORT

Inte Application No
PLI/TB 95/01167A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/53 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KADER, J.-C. AND P. MAZLIAK (ED.). PLANT LIPID METABOLISM; 11TH INTERNATIONAL MEETING ON PLANT LIPIDS, PARIS, FRANCE, JUNE 26-JULY 1, 1994. XX+588P. KLUWER ACADEMIC PUBLISHERS: DORDRECHT, NETHERLANDS; NORWELL, MASSACHUSETTS, USA. 0 (0). 1995. 509-511. ISBN: 0-7923-3250-4, XP000569979 GALLE A-M, ET AL.: "Solubilization of DELTA-12- and DELTA-6-desaturases from seeds of borage microsomes." see the whole document --- -/--	1-28

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

4 July 1996

Date of mailing of the international search report

23.07.96

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Maddox, A

INTERNATIONAL SEARCH REPORT

International Application No.

PLT/IB 95/01167

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KADER, J.-C. AND P. MAZLIAK (ED.). PLANT LIPID METABOLISM; 11TH INTERNATIONAL MEETING ON PLANT LIPIDS, PARIS, FRANCE, JUNE 26-JULY 1, 1994. XX+588P. KLUWER ACADEMIC PUBLISHERS: DORDRECHT, NETHERLANDS; NORWELL, MASSACHUSETTS, USA. 0 (0). 1995. 21-23. ISBN:0-7923-3250-4, XP000569981 SCHMIDT H, ET AL.: "PCR-based cloning of membrane-bound desaturases" see the whole document ---	1-3
Y	WO,A,93 06712 (RHONE POULENC AGROCHIMIE) 15 April 1993 see the whole document ---	4-28
A	BIOCHEM J 252 (3). 1988. 641-648. , XP000568812 GRIFFITHS G: "DELTA-6 AND DELTA-12 DESATURASE ACTIVITIES AND PHOSPHATIDIC ACID FORMATION IN MICROSOMAL PREPARATIONS FROM THE DEVELOPING COTYLEDONS OF COMMON BORAGE BORAGO-OFFICINALIS." see the whole document ---	1-3
A	BIOCHIM BIOPHYS ACTA 1158 (1). 1993. 52-58., XP002007452 GALLE A M, ET AL.: "BIOSYNTHESIS OF GAMMA LINOLENIC ACID IN DEVELOPING SEEDS OF BORAGE BORAGO-OFFICINALIS L. " see the whole document ---	1-3
A	PLANT MOLECULAR BIOLOGY, vol. 26, 1994, pages 631-642, XP002000999 SCHMIDT, H., ET AL.: "Purification and PCR-based cDNA cloning of a plastidial n-6 desaturase" see the whole document ---	1-3
A	JOURNAL OF THE AMERICAN OIL CHEMISTS SOCIETY, vol. 67, no. 4, April 1990, pages 217-225, XP002001000 BAFOR, M., ET AL.: "Properties of the glycerol acylating enzymes in microsomal preparations from the developing seeds of safflower (Carthamus tinctorius) and turnip rape (Brassica campestris) and their ability to assemble cocoa-butter type fats" see page 224, right-hand column, paragraph 2 ---	19-24

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INTERNATIONAL SEARCH REPORT

Int. Application No.
PLT/IB 95/01167

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,94 18337 (MONSANTO CO ;UNIV MICHIGAN (US); GIBSON SUSAN IRMA (US); KISHORE G) 18 August 1994 see page 32 - page 35; claim 15 ---	26-28
A	NATURE, vol. 347, 13 September 1990, pages 200-203, XP002001001 WADA, H., ET AL.: "Enhancement of chilling tolerance of a cyanobacterium by genetic manipulation of fatty acid desaturation" see the whole document ---	26-28
A	PLANT PHYSIOLOGY, vol. 105, no. 2, June 1994, pages 601-605, XP002001002 KODAMA, H., ET AL.: "Genetic enhancement of cold tolerance by expression of a gene for chloroplast omega-3 fatty acid desaturase in transgenic tobacco" see the whole document -----	26-28

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/IB 95/01167

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9306712	15-04-93	AU-B- 667848	18-04-96
		AU-B- 2881292	03-05-93
		BG-A- 98695	31-05-95
		BR-A- 9206613	11-04-95
		CA-A- 2120629	15-04-93
		CZ-A- 9400817	13-09-95
		EP-A- 0666918	16-08-95
		HU-A- 69781	28-09-95
		JP-T- 7503605	20-04-95
		NZ-A- 244685	27-06-94
ZA-A- 9207777	21-04-93		

WO-A-9418337	18-08-94	EP-A- 0684998	06-12-95

**VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT
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INTERNATIONALER RECHERCHENBERICHT

(Artikel 18 sowie Regeln 43 und 44 PCT)

Aktenzeichen des Anmelders oder Anwalts 0050/050461	WEITERES VORGEHEN siehe Mitteilung über die Übermittlung des internationalen Recherchenberichts (Formblatt PCT/ISA/220) sowie, soweit zutreffend, nachstehender Punkt 5	
Internationales Aktenzeichen PCT/EP 00/ 06223	Internationales Anmeldedatum (Tag/Monat/Jahr) 04/07/2000	(Frühestes) Prioritätsdatum (Tag/Monat/Jahr) 06/07/1999
Anmelder BASF AKTIENGESELLSCHAFT		

Dieser internationale Recherchenbericht wurde von der Internationalen Recherchenbehörde erstellt und wird dem Anmelder gemäß Artikel 18 übermittelt. Eine Kopie wird dem Internationalen Büro übermittelt.

Dieser internationale Recherchenbericht umfaßt insgesamt 3 Blätter.



Darüber hinaus liegt ihm jeweils eine Kopie der in diesem Bericht genannten Unterlagen zum Stand der Technik bei.

1. Grundlage des Berichts

- a. Hinsichtlich der **Sprache** ist die internationale Recherche auf der Grundlage der internationalen Anmeldung in der Sprache durchgeführt worden, in der sie eingereicht wurde, sofern unter diesem Punkt nichts anderes angegeben ist.



Die internationale Recherche ist auf der Grundlage einer bei der Behörde eingereichten Übersetzung der internationalen Anmeldung (Regel 23.1 b)) durchgeführt worden.

- b. Hinsichtlich der in der internationalen Anmeldung offenbaren **Nucleotid- und/oder Aminosäuresequenz** ist die internationale Recherche auf der Grundlage des Sequenzprotokolls durchgeführt worden, das



in der internationalen Anmeldung in Schriftlicher Form enthalten ist.



zusammen mit der internationalen Anmeldung in computerlesbarer Form eingereicht worden ist.



bei der Behörde nachträglich in schriftlicher Form eingereicht worden ist.



bei der Behörde nachträglich in computerlesbarer Form eingereicht worden ist.



Die Erklärung, daß das nachträglich eingereichte schriftliche Sequenzprotokoll nicht über den Offenbarungsgehalt der internationalen Anmeldung im Anmeldezeitpunkt hinausgeht, wurde vorgelegt.



Die Erklärung, daß die in computerlesbarer Form erfaßten Informationen dem schriftlichen Sequenzprotokoll entsprechen, wurde vorgelegt.

2.



Bestimmte Ansprüche haben sich als nicht recherchierbar erwiesen (siehe Feld I).

3.



Mangelnde Einheitlichkeit der Erfindung (siehe Feld II).

4. Hinsichtlich der Bezeichnung der Erfindung



wird der vom Anmelder eingereichte Wortlaut genehmigt.



wurde der Wortlaut von der Behörde wie folgt festgesetzt:

5. Hinsichtlich der Zusammenfassung



wird der vom Anmelder eingereichte Wortlaut genehmigt.



wurde der Wortlaut nach Regel 38.2b) in der in Feld III angegebenen Fassung von der Behörde festgesetzt. Der Anmelder kann der Behörde innerhalb eines Monats nach dem Datum der Absendung dieses internationalen Recherchenberichts eine Stellungnahme vorlegen.

6. Folgende Abbildung der Zeichnungen ist mit der Zusammenfassung zu veröffentlichen: Abb. Nr. _____



wie vom Anmelder vorgeschlagen



weil der Anmelder selbst keine Abbildung vorgeschlagen hat.



weil diese Abbildung die Erfindung besser kennzeichnet.



keine der Abb.



A. KLASSIFIZIERUNG DES ANMELDUNGSGEGENSTANDES

IPK 7 C12N15/82 C12N9/02 C12N15/53 C12P7/64 C11C3/00
 A01H5/00 A01H13/00 A01H15/00 A23L1/30 A23K1/16
 A61K35/78

Nach der Internationalen Patentklassifikation (IPK) oder nach der nationalen Klassifikation und der IPK

B. RESEARCHIERTE GEBIETE

Recherchierter Mindestprüfstoff (Klassifikationssystem und Klassifikationssymbole)

IPK 7 C12N C12P

Recherchierte aber nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Gebiete fallen

Während der internationalen Recherche konsultierte elektronische Datenbank (Name der Datenbank und evtl. verwendete Suchbegriffe)

EPO-Internal, WPI Data, PAJ, EMBASE, BIOSIS, MEDLINE, SCISEARCH, STRAND

C. ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie*	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
P,X	SPERLING, P. ET AL.: "A bifunctional delta-6-fatty acyl acetylenase/desaturase from thr moss <i>Ceratodon purpureus</i> " EUROPEAN JOURNAL OF BIOCHEMISTRY, Bd. 267, Juni 2000 (2000-06), Seiten 3801-3811, XP000941309 das ganze Dokument	1-4,7-11
X	GIRKE, T. ET AL.: "Identification of a novel delta-6-acyl-group desaturase by targeted gene disruption in <i>Physcomitrella patens</i> " THE PLANT JOURNAL, Bd. 15, Nr. 1, Juli 1998 (1998-07), Seiten 39-48, XP000881712 in der Anmeldung erwähnt	1-4,7-11
Y	das ganze Dokument	5,6



Weitere Veröffentlichungen sind der Fortsetzung von Feld C zu entnehmen



Siehe Anhang Patentfamilie

* Besondere Kategorien von angegebenen Veröffentlichungen :

A Veröffentlichung, die den allgemeinen Stand der Technik definiert, aber nicht als besonders bedeutsam anzusehen ist

E älteres Dokument, das jedoch erst am oder nach dem internationalen Anmeldedatum veröffentlicht worden ist

L Veröffentlichung, die geeignet ist, einen Prioritätsanspruch zweifelhaft erscheinen zu lassen, oder durch die das Veröffentlichungsdatum einer anderen im Recherchenbericht genannten Veröffentlichung belegt werden soll oder die aus einem anderen besonderen Grund angegeben ist (wie ausgeführt)

O Veröffentlichung, die sich auf eine mündliche Offenbarung, eine Benutzung, eine Ausstellung oder andere Maßnahmen bezieht

P Veröffentlichung, die vor dem internationalen Anmeldedatum, aber nach dem beanspruchten Prioritätsdatum veröffentlicht worden ist

T Spätere Veröffentlichung, die nach dem internationalen Anmeldedatum oder dem Prioritätsdatum veröffentlicht worden ist und mit der Anmeldung nicht kollidiert, sondern nur zum Verständnis des der Erfindung zugrundeliegenden Prinzips oder der ihr zugrundeliegenden Theorie angegeben ist

X Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann allein aufgrund dieser Veröffentlichung nicht als neu oder auf erfinderischer Tätigkeit beruhend betrachtet werden

Y Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann nicht als auf erfinderischer Tätigkeit beruhend betrachtet werden, wenn die Veröffentlichung mit einer oder mehreren anderen Veröffentlichungen dieser Kategorie in Verbindung gebracht wird und diese Verbindung für einen Fachmann naheliegend ist

Z Veröffentlichung, die Mitglied derselben Patentfamilie ist

Datum des Abschlusses der internationalen Recherche

9. November 2000

Absendedatum des internationalen Recherchenberichts

24/11/2000

Name und Postanschrift der Internationalen Recherchenbehörde

Europäisches Patentamt, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
 Fax: (+31-70) 340-3016

Bevollmächtigter Bediensteter

Donath, C



C.(Fortsetzung) ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie°	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
X	WO 98 46764 A (CALGENE LLC) 22. November 1998 (1998-11-22) in der Anmeldung erwähnt	11,12
Y	Seite 5, Zeile 27 -Seite 6, Zeile 17 Seite 8, Zeile 19 -Seite 36, Zeile 27; Beispiele 6-8,13,14,16 ---	1-10
X	WO 99 27111 A (UNIVERSITY OF BRISTOL) 3. Juni 1999 (1999-06-03) in der Anmeldung erwähnt	11
Y	Seite 4, Zeile 7 -Seite 9, Zeile 28; Beispiele 1,2 ---	1-10
X	SAYANOVA, O. ET AL.: "Expression of a borage desaturase cDNA containing an N-terminal cytochrome b5 domain results in the accumulation of high levels of delta-6-desaturated fatty acids in transgenic tobacco" PROC.NATL.ACAD.SCI.USA, Bd. 94, April 1997 (1997-04), Seiten 4211-4216, XP002099447 in der Anmeldung erwähnt	11
Y	das ganze Dokument ---	1-10
X	WO 96 21022 A (RHONE-POULENC AGROCHIMIE) 11. Juli 1996 (1996-07-11) in der Anmeldung erwähnt	11
Y	Seite 3, Zeile 3 - Zeile 23 Seite 5, Zeile 16 -Seite 19, Zeile 24; Beispiele 6,13,14 -----	1-10



INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PC 00/06223

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9846764	A	22-10-1998	US 5972664 A	26-10-1999
			US 6075183 A	13-06-2000
			US 5968809 A	19-10-1999
			US 6051754 A	18-04-2000
			AU 720677 B	08-06-2000
			AU 7114798 A	11-11-1998
			AU 720725 B	08-06-2000
			AU 7114898 A	11-11-1998
			BG 103796 A	31-05-2000
			BG 103798 A	31-05-2000
			BR 9808506 A	23-05-2000
			BR 9809083 A	01-08-2000
			CN 1253587 T	17-05-2000
			CN 1253588 T	17-05-2000
			EP 0996732 A	03-05-2000
			EP 1007691 A	14-06-2000
			NO 994924 A	30-11-1999
			NO 994926 A	30-11-1999
			PL 336067 A	05-06-2000
			PL 336077 A	05-06-2000
			WO 9846765 A	22-10-1998
			AU 6961698 A	11-11-1998
			BG 103797 A	28-04-2000
			BR 9808507 A	23-05-2000
			CN 1252099 T	03-05-2000
			EP 0975766 A	02-02-2000
			NO 994925 A	30-11-1999
			PL 336143 A	05-06-2000
			WO 9846763 A	22-10-1998
WO 9927111	A	03-06-1999	AU 1249799 A	15-06-1999
			EP 1032682 A	06-09-2000
			ZA 9810716 A	16-06-1999
WO 9621022	A	11-07-1996	US 5614393 A	25-03-1997
			AU 707061 B	01-07-1999
			AU 4673596 A	24-07-1996
			BR 9510411 A	19-05-1998
			CA 2207906 A	11-07-1996
			CN 1177379 A	25-03-1998
			EP 0801680 A	22-10-1997
			JP 10511848 T	17-11-1998
			US 5789220 A	04-08-1998



VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS

PCT

REC'D 24 OCT 2001

INTERNATIONALER VORLÄUFIGER PRÜFUNGSBERICHT

WIPO PCT

(Artikel 36 und Regel 70 PCT)



716

Aktenzeichen des Anmelders oder Anwalts 0050/050461	WEITERES VORGEHEN siehe Mitteilung über die Übersendung des internationalen vorläufigen Prüfungsberichts (Formblatt PCT/IPEA/416)	
Internationales Aktenzeichen PCT/EP00/06223	Internationales Anmeldedatum (Tag/Monat/Jahr) 04/07/2000	Prioritätsdatum (Tag/Monat/Tag) 06/07/1999
Internationale Patentklassifikation (IPK) oder nationale Klassifikation und IPK C12N15/82		
Anmelder BASF PLANT SCIENCE GMBH et al.		

1. Dieser internationale vorläufige Prüfungsbericht wurde von der mit der internationalen vorläufigen Prüfung beauftragten Behörde erstellt und wird dem Anmelder gemäß Artikel 36 übermittelt.
2. Dieser BERICHT umfaßt insgesamt 6 Blätter einschließlich dieses Deckblatts.
☐ Außerdem liegen dem Bericht ANLAGEN bei; dabei handelt es sich um Blätter mit Beschreibungen, Ansprüchen und/oder Zeichnungen, die geändert wurden und diesem Bericht zugrunde liegen, und/oder Blätter mit vor dieser Behörde vorgenommenen Berichtigungen (siehe Regel 70.16 und Abschnitt 607 der Verwaltungsrichtlinien zum PCT).
Diese Anlagen umfassen insgesamt Blätter.

3. Dieser Bericht enthält Angaben zu folgenden Punkten:

- I ☒ Grundlage des Berichts
- II ☐ Priorität
- III ☐ Keine Erstellung eines Gutachtens über Neuheit, erfinderische Tätigkeit und gewerbliche Anwendbarkeit
- IV ☐ Mangelnde Einheitlichkeit der Erfindung
- V ☒ Begründete Feststellung nach Artikel 35(2) hinsichtlich der Neuheit, der erfinderischen Tätigkeit und der gewerblichen Anwendbarkeit; Unterlagen und Erklärungen zur Stützung dieser Feststellung
- VI ☐ Bestimmte angeführte Unterlagen
- VII ☐ Bestimmte Mängel der internationalen Anmeldung
- VIII ☒ Bestimmte Bemerkungen zur internationalen Anmeldung

Datum der Einreichung des Antrags 11/12/2000	Datum der Fertigstellung dieses Berichts 19.10.2001
Name und Postanschrift der mit der internationalen vorläufigen Prüfung beauftragten Behörde:  Europäisches Patentamt D-80298 München Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Bevollmächtigter Bediensteter Donath, C Tel. Nr. +49 89 2399 8710 



INTERNATIONALER VORLÄUFIGER PRÜFUNGSBERICHT

Internationales Aktenzeichen PCT/EP00/06223

I. Grundlage des Berichts

1. Hinsichtlich der **Bestandteile** der internationalen Anmeldung (*Ersatzblätter, die dem Anmeldeamt auf eine Aufforderung nach Artikel 14 hin vorgelegt wurden, gelten im Rahmen dieses Berichts als "ursprünglich eingereicht" und sind ihm nicht beigefügt, weil sie keine Änderungen enthalten (Regeln 70.16 und 70.17)*):
Beschreibung, Seiten:

1-34 ursprüngliche Fassung

Patentansprüche, Nr.:

1-12 ursprüngliche Fassung

Sequenzprotokoll in der Beschreibung, Seiten:

1-6, in der ursprünglich eingereichten Fassung.

2. Hinsichtlich der **Sprache**: Alle vorstehend genannten Bestandteile standen der Behörde in der Sprache, in der die internationale Anmeldung eingereicht worden ist, zur Verfügung oder wurden in dieser eingereicht, sofern unter diesem Punkt nichts anderes angegeben ist.

Die Bestandteile standen der Behörde in der Sprache: zur Verfügung bzw. wurden in dieser Sprache eingereicht; dabei handelt es sich um

- ☐ die Sprache der Übersetzung, die für die Zwecke der internationalen Recherche eingereicht worden ist (nach Regel 23.1(b)).
- ☐ die Veröffentlichungssprache der internationalen Anmeldung (nach Regel 48.3(b)).
- ☐ die Sprache der Übersetzung, die für die Zwecke der internationalen vorläufigen Prüfung eingereicht worden ist (nach Regel 55.2 und/oder 55.3).

3. Hinsichtlich der in der internationalen Anmeldung offenbarten **Nucleotid- und/oder Aminosäuresequenz** ist die internationale vorläufige Prüfung auf der Grundlage des Sequenzprotokolls durchgeführt worden, das:

- ☒ in der internationalen Anmeldung in schriftlicher Form enthalten ist.
- ☒ zusammen mit der internationalen Anmeldung in computerlesbarer Form eingereicht worden ist.
- ☐ bei der Behörde nachträglich in schriftlicher Form eingereicht worden ist.
- ☐ bei der Behörde nachträglich in computerlesbarer Form eingereicht worden ist.
- ☐ Die Erklärung, daß das nachträglich eingereichte schriftliche Sequenzprotokoll nicht über den Offenbarungsgehalt der internationalen Anmeldung im Anmeldezeitpunkt hinausgeht, wurde vorgelegt.
- ☐ Die Erklärung, daß die in computerlesbarer Form erfassten Informationen dem schriftlichen Sequenzprotokoll entsprechen, wurde vorgelegt.

4. Aufgrund der Änderungen sind folgende Unterlagen fortgefallen:

☐ Beschreibung, Seiten:



INTERNATIONALER VORLÄUFIGER PRÜFUNGSBERICHT

Internationales Aktenzeichen PCT/EP00/06223

- ☐ Ansprüche, Nr.:
☐ Zeichnungen, Blatt:

5. ☐ Dieser Bericht ist ohne Berücksichtigung (von einigen) der Änderungen erstellt worden, da diese aus den angegebenen Gründen nach Auffassung der Behörde über den Offenbarungsgehalt in der ursprünglich eingereichten Fassung hinausgehen (Regel 70.2(c)).

(Auf Ersatzblätter, die solche Änderungen enthalten, ist unter Punkt 1 hinzuweisen; sie sind diesem Bericht beizufügen).

6. Etwaige zusätzliche Bemerkungen:

V. Begründete Feststellung nach Artikel 35(2) hinsichtlich der Neuheit, der erfinderischen Tätigkeit und der gewerblichen Anwendbarkeit; Unterlagen und Erklärungen zur Stützung dieser Feststellung

1. Feststellung

Neuheit (N)	Ja: Ansprüche	5,6
	Nein: Ansprüche	1-4,7-12
Erfinderische Tätigkeit (ET)	Ja: Ansprüche	
	Nein: Ansprüche	1-12
Gewerbliche Anwendbarkeit (GA)	Ja: Ansprüche	1-12
	Nein: Ansprüche	

2. Unterlagen und Erklärungen
siehe Beiblatt

VIII. Bestimmte Bemerkungen zur internationalen Anmeldung

Zur Klarheit der Patentansprüche, der Beschreibung und der Zeichnungen oder zu der Frage, ob die Ansprüche in vollem Umfang durch die Beschreibung gestützt werden, ist folgendes zu bemerken:
siehe Beiblatt



Ad section V.:

1. Auf folgende Dokumente wird in diesem Bescheid Bezug genommen:

D1 The plant Journal 15(1), 39-48, 1998

D2 WO-A-98/46764

D3 WO-A-96/21022

2. Die vorliegende Internationale Anmeldung betrifft ein Verfahren zur Herstellung von ungesättigten Fettsäuren. Es werden transgene Organismen hergestellt (vorzugsweise Pflanzen, Algen oder Pilze), die aufgrund der Expression einer delta-6-Desaturase aus Moos einen erhöhten Gehalt an Fettsäuren, Ölen oder Lipiden mit delta-6-Doppelbindungen aufweisen. Desweiteren betrifft die Internationale Anmeldung die für das obige Verfahren hergestellten transgenen Organismen, die durch das Verfahren hergestellten Öle, Lipide oder Fettsäuren, sowie deren Verwendung in Futtermitteln, Nahrungsmitteln, Kosmetika oder Pharmazeutika.

Im Hinblick auf die im Internationalen Recherchenbericht zitierten Dokumente können nur die Ansprüche 5 und 6 der vorliegenden Internationalen Anmeldung als neu betrachtet werden (Artikel 33(2) PCT).

- 2.1 D1 offenbart die Isolierung und Klonierung einer cDNA sowie der dazu korrespondierenden genomischen DNA-Sequenz aus dem Moos *Physcomitrella patens*. Das von dieser DNA kodierte Protein wurde als eine delta-6-Desaturase identifiziert. Durch Expression des Proteins in *S.cerevisiae* sowie durch Analyse der aus diesem transgenen Organismus gewonnenen Fettsäuren konnte bestätigt werden, daß die klonierte DNA für eine delta-6-Desaturase kodiert. Sowohl die Nukleotid-Sequenz als auch die Aminosäure-Sequenz der in D1 isolierten DNA bzw. des korrespondierenden Proteins weisen eine 100 %ige Identität über die gesamte Länge mit der in der vorliegenden Internationalen Anmeldung offenbarten Sequenz SEQ ID NO:1 bzw. SEQ ID NO:2 auf (s.D1,S.44-47, 'Functional expression of PPDES6 in *Saccharomyces cerevisiae*', 'Discussion', 'Expression in *S.cerevisiae*', 'Lipid analysis' and Fig.1).

Das obige Dokument ist daher neuheitsschädlich für den Gegenstand der



Ansprüche 1-4 und 7-11.

- 2.2 D2 beschreibt eine Methode zur Herstellung von mehrfach ungesättigten langkettigen Fettsäuren in Pflanzen. Expressionskonstrukte enthaltend DNA-Sequenzen kodierend für eine delta-6-, delta-5- oder delta-12-Desaturase wurden zunächst zur Herstellung dieser transgenen Pflanzen verwendet. Es wurde gezeigt, daß eine Expression dieser Desaturasen in den Pflanzen die Herstellung von großen Mengen an mehrfach ungesättigten Fettsäuren ermöglicht, und auf diese Weise zu einer Veränderung des Fettsäure-Profils dieser Pflanzen führt. Diese Manipulation des Fettsäure-Profils erlaubt nunmehr die Herstellung von kommerziell nutzbaren Mengen an Pflanzenölen sowie deren Verwendung als Pharmazeutika, Nahrungsmittel etc. (s.D2, S.5, Zeile 27 - S.6, Zeile 17, S.8, Zeile 19 - S.36, Zeile 27, Beispiele 6-8,13,14,16).

In Hinblick auf D2 ist der Gegenstand der Ansprüche 11 und 12 daher nicht neu.

- 2.3 D3 offenbart die Klonierung einer DNA kodierend für eine delta-6-Desaturase aus dem Cyanobakterium *Synechocystis* sowie einer cDNA kodierend für eine delta-6-Desaturase aus *Borretsch*. Diese DNA-Sequenzen wurden in verschiedenen Organismen, wie z.B. in Tabakpflanzen, exprimiert, und es wurde gezeigt, daß in den transgenen Organismen mittels dieser Sequenzen ungesättigte Fettsäuren, wie z.B. GLA, hergestellt wurden (s.D3, S.3, Zeilen 3-23, S.5. Zeile 16 - S.19, Zeile 24, Beispiele 6,13,14, Ansprüche 11-18).

Das obige Dokument ist daher neuheitsschädlich für den Gegenstand des Anspruches 11.

3. Zur Beurteilung eines erfinderischen Schrittes der Ansprüche 5 und 6 der vorliegenden Internationalen Anmeldung wird ebenfalls D1 als der nächstliegende Stand der Technik herangezogen.
Diese Ansprüche betreffen die Verwendung einer transgenen Alge oder Pflanze, insbesondere einer Ölfuchtpflanze im Verfahren zur Herstellung von ungesättigten Fettsäuren.
Diese abhängigen Ansprüche scheinen keine zusätzlichen Merkmale zu enthalten, welche in Kombination mit den Merkmalen der Ansprüche auf die sie



sich beziehen, einen erfinderischen Schritt beinhalten. Die Verwendung transgener Pflanzen, bzw. Ölfuchtpflanzen, in einem Verfahren zur Herstellung von ungesättigten Fettsäuren ist bereits aus D2 oder D3 bekannt.

Der Gegenstand der Ansprüche 5 und 6 beruht daher nicht auf einer nach Artikel 33(3) PCT erforderlichen erfinderischen Tätigkeit.

Ad section VIII.:

1. Den Ansprüchen 1,4 und 7-9 mangelt es an Klarheit aufgrund der Ausdrücke "Organismus" und "Tiere". Die Beschreibung der vorliegenden internationalen Anmeldung nimmt nur Bezug auf tierische Zellen, nicht jedoch auf Tiere als solche. Desweiteren ist es absolut notwendig klarzustellen, daß der Mensch **nicht** unter die Begriffe "Organismus" und "Tiere" fällt.



(12) NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES
PATENTWESENS (PCT) VERÖFFENTLICHTE INTERNATIONALE ANMELDUNG

(19) Weltorganisation für geistiges Eigentum
Internationales Büro



(43) Internationales Veröffentlichungsdatum
11. Januar 2001 (11.01.2001)

PCT

(10) Internationale Veröffentlichungsnummer
WO 01/02591 A1

(51) Internationale Patentklassifikation⁷: C12N 15/82,
9/02, 15/53, C12P 7/64, C11C 3/00, A01H 5/00, 13/00,
15/00, A23L 1/30, A23K 1/16, A61K 35/78

(74) Gemeinsamer Vertreter: BASF AKTIENGE-
SELLSCHAFT; 67056 Ludwigshafen (DE).

(21) Internationales Aktenzeichen: PCT/EP00/06223

(22) Internationales Anmeldedatum:
4. Juli 2000 (04.07.2000)

(25) Einreichungssprache: Deutsch

(26) Veröffentlichungssprache: Deutsch

(30) Angaben zur Priorität:
09/347.531 6. Juli 1999 (06.07.1999) US
100 30 976.3 30. Juni 2000 (30.06.2000) DE

(81) Bestimmungsstaaten (*national*): AE, AG, AL, AM, AT,
AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU,
CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM,
HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK,
LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX,
MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL,
TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Bestimmungsstaaten (*regional*): ARIPO-Patent (GH,
GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), eura-
sisches Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
europäisches Patent (AT, BE, CH, CY, DE, DK, ES, FI,
FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI-Patent
(BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE,
SN, TD, TG).

(71) Anmelder (*für alle Bestimmungsstaaten mit Ausnahme
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(72) Erfinder; und

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Country Road 228, Oxford, MS 38655 (US). DA COSTA
E SILVA, Oswaldo [BR/US]; 203 Littleford Lane, Apex,
NC 27502 (US).

Veröffentlicht:

- Mit internationalem Recherchenbericht.
- Vor Ablauf der für Änderungen der Ansprüche geltenden
Frist; Veröffentlichung wird wiederholt, falls Änderungen
eintreffen.

Zur Erklärung der Zweibuchstaben-Codes, und der anderen
Abkürzungen wird auf die Erklärungen ("Guidance Notes on
Codes and Abbreviations") am Anfang jeder regulären Ausgabe
der PCT-Gazette verwiesen.

50461

020323

(54) Title: PLANTS EXPRESSING $\Delta 6$ -DESATURASE GENES AND OILS FROM THESE PLANTS CONTAINING PUFAS
AND METHOD FOR PRODUCING UNSATURATED FATTY ACIDS

(54) Bezeichnung: $\Delta 6$ -DESATURASEGENE EXPRIMIERENDE PFLANZEN UND PUFAS ENTHALTENDE ÖLE AUS DIE-
SEN PFLANZEN UND EIN VERFAHREN ZUR HERSTELLUNG UNGESÄTTIGTER FETTSÄUREN

(57) Abstract: The invention relates to an improved method for producing unsaturated fatty acids and to a method for producing
triglycerides with an increased unsaturated fatty acid content. The invention also relates to the production of a transgenic organism,
preferably a transgenic plant or a transgenic micro-organism, containing increased quantities of unsaturated fatty acids, oils or lipids
with $\Delta 6$ -double bonds as a result of the expression of a $\Delta 6$ -desaturase, from moss. The invention also relates to transgenic organ-
isms containing a $\Delta 6$ -desaturase gene, and to the use of the unsaturated fatty acids or triglycerides with an increased unsaturated
fatty acid content produced in the method.

(57) Zusammenfassung: Die vorliegende Erfindung betrifft ein verbessertes Verfahren zur Herstellung von ungesättigten Fettsäu-
ren sowie ein Verfahren zur Herstellung von Triglyceriden mit einem erhöhten Gehalt an ungesättigten Fettsäuren. Die Erfindung
betrifft die Herstellung eines transgenen Organismus bevorzugt einer transgenen Pflanze oder eines transgenen Mikroorganismus
mit erhöhtem Gehalt an Fettsäuren, Ölen oder Lipiden mit $\Delta 6$ -Doppelbindungen aufgrund der Expression einer $\Delta 6$ -Desaturase aus
Moos. Ausserdem betrifft die Erfindung transgene Organismen, die ein $\Delta 6$ -Desaturasegen enthalten, sowie die Verwendung der im
Verfahren hergestellten ungesättigten Fettsäuren bzw. Triglyceride mit einem erhöhten Gehalt an ungesättigten Fettsäuren.

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$\Delta 6$ -Desaturasegene exprimierende Pflanzen und PUFAS enthaltende Öle aus diesen Pflanzen und ein Verfahren zur Herstellung ungesättigter Fettsäuren

5

Beschreibung

Die vorliegende Erfindung betrifft ein verbessertes Verfahren zur Herstellung von ungesättigten Fettsäuren sowie ein Verfahren
10 zur Herstellung von Triglyceriden mit einem erhöhten Gehalt an ungesättigten Fettsäuren. Die Erfindung betrifft die Herstellung eines transgenen Organismus bevorzugt einer transgenen Pflanze oder eines transgenen Mikroorganismus mit erhöhtem Gehalt an Fettsäuren, Ölen oder Lipiden mit $\Delta 6$ -Doppelbindungen aufgrund
15 der Expression einer $\Delta 6$ -Desaturase aus Moos.

Außerdem betrifft die Erfindung transgene Organismen, die ein $\Delta 6$ -Desaturasegen enthalten, sowie die Verwendung der im Verfahren hergestellten ungesättigten Fettsäuren bzw. Triglyceride mit
20 einem erhöhten Gehalt an ungesättigten Fettsäuren.

Fettsäuren und Triglyceride haben eine Vielzahl von Anwendungen in der Lebensmittelindustrie, der Tierernährung, der Kosmetik und im Pharmabereich. Je nachdem ob es sich um freie gesättigte oder
25 ungesättigte Fettsäuren oder um Triglyceride mit einem erhöhten Gehalt an gesättigten oder ungesättigten Fettsäuren handelt, sind sie für die unterschiedlichsten Anwendungen geeignet, so werden beispielsweise mehrfach ungesättigte Fettsäuren Babynahrung zur Erhöhung des Nährwertes zugesetzt. Hauptsächlich werden die ver-
30 schiedenen Fettsäuren und Triglyceride aus Mikroorganismen wie Mortierella oder aus Öl-produzierenden Pflanzen wie Soja, Raps, Sonnenblume und weiteren gewonnen, wobei sie in der Regel in Form ihrer Triacylglyceride anfallen. Sie können aber auch aus Tieren wie Fischen gewonnen werden. Die freien Fettsäuren werden vor-
35 teilhaft durch Verseifung hergestellt.

Je nach Anwendungszweck sind Öle mit gesättigten oder ungesättigten Fettsäuren bevorzugt, so sind z.B. in der humanen Ernährung Lipide mit ungesättigten Fettsäuren speziell mehrfach ungesättig-
40 ten Fettsäuren bevorzugt, da sie einen positiven Einfluß auf den Cholesterinspiegel im Blut und damit auf die Möglichkeit einer Herzerkrankung haben. Auch eine positive Wirkung auf die Carcinogenese wird den ungesättigten Fettsäuren zugeschrieben. Sie sind außerdem wichtige Ausgangsstoffe für die Synthese von
45 Verbindungen, die wichtige biologische Vorgänge innerhalb des

Organismus steuern. Sie finden deshalb in verschiedenen diätischen Lebensmitteln oder Medikamenten Anwendung.

Aufgrund ihrer positiven Eigenschaften hat es in der Vergangenheit nicht an Ansätzen gefehlt, Gene, die an der Synthese von Fettsäuren bzw. Triglyceriden beteiligt sind, für die Herstellung von Ölen in verschiedenen Organismen mit geändertem Gehalt an ungesättigten Fettsäuren verfügbar zu machen. So wird in WO 91/13972 und seinem US-Äquivalent eine Δ^9 -Desaturase beschrieben. In WO 93/11245 wird eine Δ^{15} -Desaturase in WO 94/11516 wird eine Δ^{12} -Desaturase beansprucht. Δ^6 -Desaturasen werden in Girke et al. (The Plant Journal, 15, 1998: 39-48), Napier et al. (Biochem. J., 330, 1998: 611-614), Murata et al. (Biosynthesis of γ -linolenic acid in cyanobacterium *Spirulina patensis*, pp 22-32, In: γ -linolenic acid, metabolism and its roles in nutrition and medicine, Huang, Y. and Milles, D.E. [eds.], AOC Press, Champaign, Illinois), Sayanova et al. (Proc. Natl. Acad. Sci. USA, 94, 1997: 4211-4216), WO 98/46764, Cho et al. (J. Biol. Chem., 274, 1999: 471-477), Aki et al. (Biochem. Biophys. Res. Commun., 255, 1999: 575-579), und Reddy et al. (Plant Mol. Biol., 27, 1993: 293-300) beschrieben. Weitere Desaturasen werden beispielsweise in EP-A-0 550 162, WO 94/18337, WO 97/30582, WO 97/21340, WO 95/18222, EP-A-0 794 250, Stukey et al., J. Biol. Chem., 265, 1990: 20144-20149, Wada et al., Nature 347, 1990: 200-203 oder Huang et al., Lipids 34, 1999: 649-659 beschrieben. Weitere Δ^6 -Desaturasen werden in WO 93/06712, US 5,614,393, US5,614,393, WO 96/21022, WO00/21557 und WO 99/27111 beschrieben. Die biochemische Charakterisierung der verschiedenen Desaturasen ist jedoch bisher nur unzureichend erfolgt, da die Enzyme als membrangebundene Proteine nur sehr schwer zu isolieren und charakterisieren sind (McKeon et al., Methods in Enzymol. 71, 1981: 12141-12147, Wang et al., Plant Physiol. Biochem., 26, 1988: 777-792). In der Regel erfolgt die Charakterisierung membrangebundener Desaturasen durch Einbringung in einen geeigneten Organismus, der anschließend auf Enzymaktivität mittels Edukt- und Produktanalyse untersucht wird. Die Anwendung zur Produktion in transgenen Organismen beschrieben wie in WO 98/46763 WO98/46764, WO98/46765. Dabei wird auch die Expression verschiedener Desaturasen wie in WO99/64616 oder WO98/46776 und Bildung polyungesättigter Fettsäuren beschrieben und beansprucht. Bezüglich der Effektivität der Expression von Desaturasen und ihren Einfluß auf die Bildung polyungesättigter Fettsäuren ist anzumerken, daß durch Expression einer einzelnen Desaturase wie im vorgenannten Stand der Technik beschrieben lediglich geringe Gehalte an ungesättigten Fettsäuren beispielsweise an Δ^6 ungesättigten Fettsäuren/Lipiden wie z.B. γ -Linolensäure erreicht wurden und werden.

Nach wie vor besteht daher ein großer Bedarf an neuen und besser geeigneten Genen, die für Enzyme codieren, die an der Biosynthese ungesättigter Fettsäuren beteiligt sind und es ermöglichen, diese in einem technischen Maßstab herzustellen. Weiterhin besteht
5 nach wie vor ein Bedarf an verbesserten Verfahren zur Gewinnung möglichst hoher Gehalte an polyungesättigten Fettsäuren.

Es bestand daher die Aufgabe ein Verfahren zur Herstellung von ungesättigten Fettsäuren unter Verwendung von Genen, die
10 beispielsweise für Desaturase-Enzyme codieren und die an der Synthese mehrfach ungesättigter Fettsäuren in den Samen einer Ölseed beteiligt sind, bereitzustellen und so den Gehalt polyungesättigter Fettsäuren zu erhöhen. Diese Aufgabe wurde durch ein Verfahren zur Herstellung von ungesättigten Fettsäuren
15 gelöst, dadurch gekennzeichnet, daß mindestens eine isolierte Nukleinsäuresequenz, die für ein Polypeptid mit $\Delta 6$ -Desaturaseaktivität codiert, ausgewählt aus der Gruppe:

- a) einer Nukleinsäuresequenz mit der in SEQ ID NO: 1 dargestellten Sequenz,
20
- b) Nukleinsäuresequenzen, die sich als Ergebnis des degenerierten genetischen Codes von der in SEQ ID NO: 1 ableiten
- 25 c) Derivate der in SEQ ID NO: 1 dargestellten Nukleinsäuresequenz, die für Polypeptide mit der in SEQ ID NO: 2 dargestellten Aminosäuresequenzen codieren und mindestens 50 % Homologie auf Aminosäureebene aufweisen, ohne daß die enzymatische Wirkung der Polypeptide wesentlich reduziert
30 ist,

in einen Organismus eingebracht wird, dieser Organismus angezogen wird, wobei der angezogene Organismus mindestens 1 Mol-% ungesättigte Fettsäuren bezogen auf den gesamten Fettsäuregehalt
35 im Organismus enthält.

Unter Anzucht des Organismus ist die Kultivierung von Pflanzen ebenso zu verstehen wie die Anzucht von eukaryontischen oder prokaryontischen Mikroorganismen wie Bakterien, Hefen, Pilzen,
40 Ciliaten, Algen, Cyanobakterien, tierischen oder pflanzlichen Zellen oder Zellverbänden oder die Anzucht von Tieren.

Die in den im erfindungsgemäßen Verfahren gewonnenen Organismen enthalten in der Regel ungesättigte Fettsäuren in Form von
45 gebundenen Fettsäuren, das heißt die ungesättigten Fettsäuren liegen überwiegend in Form ihrer Mono-, Di- oder Triglyceride, Glycolipide, Lipoproteine oder Phospholipide wie Öle oder Lipide

- oder sonstig als Ester oder Amide gebundenen Fettsäuren vor. Auch freie Fettsäuren sind in den Organismen in Form der freien Fettsäuren oder in Form ihrer Salze enthalten. Die freien oder gebundenen ungesättigten Fettsäuren enthalten vorteilhaft gegen-
- 5 über den Ausgangsorganismen einen erhöhten Gehalt an Fettsäuren mit Δ^6 -Doppelbindungen wie vorteilhaft γ -Linolensäure. Die durch Anzucht im erfindungsgemäßen Verfahren gewonnenen Organismen und die in ihnen enthaltenen ungesättigten Fettsäuren können direkt beispielsweise zur Herstellung von pharmazeutischen
- 10 Zubereitungen, von Agrochemikalien, Futtermitteln oder Lebensmitteln verwendet werden oder aber nach Isolierung aus den Organismen. Dabei können alle Stufen der Aufreinigung der ungesättigten Fettsäuren verwendet werden, das heißt von Rohextrakten der Fettsäuren bis zu vollständig gereinigten Fettsäuren sind für
- 15 die Herstellung der vorgenannten Produkte geeignet. In einer vorteilhaften Ausführungsform können die gebundenen Fettsäuren aus beispielsweise den Ölen bzw. Lipiden beispielsweise über eine basische Hydrolyse z.B. mit NaOH oder KOH freigesetzt werden. Diese freien Fettsäuren können direkt im erhaltenen Gemisch oder
- 20 nach weiterer Aufreinigung zur Herstellung von pharmazeutischen Zubereitungen, von Agrochemikalien, Futtermitteln oder Lebensmitteln verwendet werden. Auch können die gebundenen oder freien Fettsäuren zur Umesterung oder Veresterung beispielsweise mit anderen Mono-, Di- oder Triglyceriden oder Glycerin verwendet
- 25 werden, um den Anteil an ungesättigten Fettsäuren in diesen Verbindungen beispielsweise in den Triglyceriden zu erhöhen.

Ein weiterer erfindungsgemäßer Gegenstand ist ein Verfahren zur Herstellung von Triglyceriden mit einem erhöhten Gehalt an un-

30 sättigten Fettsäuren, indem man Triglyceride mit gesättigten oder ungesättigten oder gesättigten und ungesättigten Fettsäuren mit mindestens einem der Protein, das durch die Sequenz SEQ ID NO: 2 codiert wird, inkubiert. Vorteilhaft wird das Verfahren in Gegenwart von Verbindungen durchgeführt, die Reduktionsäquivalente

35 aufnehmen oder abgeben können. Anschließend können die Fettsäuren aus den Triglyceriden freigesetzt werden.

Die oben genannten Verfahren ermöglichen vorteilhaft die Synthese von Fettsäuren oder gebundenen Fettsäuren wie Triglyceriden mit

40 einem erhöhten Gehalt an Fettsäuren mit Δ^6 -Doppelbindungen.

Als Organismen für die genannten Verfahren seien beispielhaft Pflanzen wie Arabidopsis, Gerste, Weizen, Roggen, Hafer, Mais, Soja, Reis, Baumwolle, Zuckerrübe, Tee, Karotte, Paprika, Canola,

45 Sonnenblume, Flachs, Hanf, Kartoffel, Triticale, Tabak, Tomate, Raps, Kaffee, Tapioka, Maniok, Pfeilwurz, Tagetes, Alfalfa, Erdnuß, Rizinus, Kokosnuß, Ölpalme, Färbersaflor (Carthamus

- tinctorius), Salat und den verschiedenen Baum-, Nuß- und Weinspezies, oder Kakaobohne, Mikroorganismen wie Pilze *Mortierella*, *Saprolegnia* oder *Pythium*, Bakterien wie die Gattung *Escherichia*, Cyanobakterien, Algen oder Protozoen wie Dinoflagellaten wie
- 5 *Cryptocodinium* genannt. Bevorzugt werden Organismen, die natürlicherweise Öle in größeren Mengen synthetisieren können wie Mikroorganismen wie Pilze wie *Mortierella alpina*, *Pythium insidiosum* oder Pflanzen wie Soja, Raps, Kokosnuß, Ölpalme, Canola, Färbersaflor (*Carthamus tinctorius*), Rizinus, Calendula,
- 10 Lein, Borretsch, Erdnuß, Kakaobohne oder Sonnenblume, besonders bevorzugt werden Soja, Raps oder Sonnenblume.

- Die in den Verfahren verwendeten Organismen werden je nach Wirtsorganismus in dem Fachmann bekannter Weise angezogen bzw.
- 15 gezüchtet. Mikroorganismen wie Bakterien, Pilze, Ciliaten, pflanzliche oder tierische Zellen werden in der Regel in einem flüssigen Medium, das eine Kohlenstoffquelle meist in Form von Zuckern, eine Stickstoffquelle meist in Form von organischen Stickstoffquellen wie Hefeextrakt oder Salzen wie Ammoniumsulfat,
- 20 Spurenelemente wie Eisen-, Mangan-, Magnesiumsalze und gegebenenfalls Vitamine enthält, bei Temperaturen zwischen 0°C und 100°C, bevorzugt zwischen 10°C bis 60°C unter je nach Organismus Sauerstoffbegasung oder in Abwesenheit von Sauerstoff angezogen. Dabei kann der pH der Nährflüssigkeit auf einen festen Wert gehalten
- 25 werden, das heißt der pH wird während der Anzucht reguliert oder der pH wird nicht reguliert und verändert sich während der Anzucht. Die Anzucht kann batch weise, semi batch weise oder kontinuierlich erfolgen. Nährstoffe können zu Beginn der Fermentation vorgelegt oder semikontinuierlich oder kontinuier-
- 30 lich nach gefüttert werden. Auch eine Anzucht auf festen Medien ist möglich.

- Pflanzen werden nach Transformation in der Regel zunächst regeneriert und anschließend wie üblich angezogen bzw. angebaut.
- 35 Dies kann im Gewächshaus oder im Freiland erfolgen.

- Aus den Organismen werden nach Anzucht die Lipide in üblicherweise gewonnen. Hierzu können die Organismen nach Ernte zunächst aufgeschlossen werden oder direkt verwendet werden. Die Lipide
- 40 werden vorteilhaft mit geeigneten Lösungsmitteln wie apolare Lösungsmittel wie Hexan oder Ethanol, Isopropanol oder Gemischen wie Hexan/Isopropanol, Phenol/Chloroform/Isoamylalkohol bei Temperaturen zwischen 0°C bis 80°C, bevorzugt zwischen 20°C bis 50°C extrahiert. Die Biomasse wird in der Regel mit einem Über-
- 45 schuß an Lösungsmittel extrahiert beispielsweise einem Überschuß von Lösungsmittel zu Biomasse von 1:4. Das Lösungsmittel wird anschließend beispielsweise über eine Destillation entfernt.

Die Extraktion kann auch mit superkritischem CO₂ erfolgen. Nach Extraktion kann die restliche Biomasse beispielsweise über Filtration entfernt werden.

- 5 Das so gewonnene Rohöl kann anschließend weiter aufgereinigt werden, beispielsweise in dem Trübungen über das Versetzen mit polaren Lösungsmittel wie Aceton oder Chloroform und anschließender Filtration oder Zentrifugation entfernt werden. Auch eine weitere Reinigung über chromatographische Verfahren,
10 Destillation oder Kristallisation ist möglich.

Zur Gewinnung der freien Fettsäuren aus den Triglyceriden werden diese in üblicher Weise, wie oben beschrieben, verseift.

- 15 Ein weiterer Gegenstand der Erfindung sind ungesättigte Fettsäuren sowie Triglyceride mit einem erhöhten Gehalt an ungesättigten Fettsäuren, die nach den oben genannten Verfahren hergestellt wurden, sowie deren Verwendung zur Herstellung von Nahrungsmitteln, Tierfutter, Kosmetika oder Pharmazeutika. Hierzu
20 werden diese den Nahrungsmitteln, dem Tierfutter, den Kosmetika oder Pharmazeutika in üblichen Mengen zugesetzt.

- Im erfindungsgemäßen Verfahren wurden durch Expression einer $\Delta 6$ -Desaturase aus Moos in Organismen wie Pilze, Bakterien,
25 Tieren oder Pflanzen, bevorzugt Pilzen, Bakterien und Pflanzen, besonders bevorzugt in Pflanzen, ganz besonders bevorzugt in Ölfruchtpflanzen wie Raps, Canola, Lein, Soja, Sonnenblume, Borretsch, Rizinus, Ölpalme, Färbersaflor (*Carthamus tinctorius*), Kokosnuß, Erdnuß oder Kakaobohne höhere Gehalte an ungesättigten
30 Fettsäuren wie γ -Linolensäure erhalten. Auch die Expression in Feldfrüchten, wie Mais, Weizen, Roggen, Hafer, Triticale, Reis, Gerste, Alfalfa, oder Buschpflanzen (Kaffee, Kakao, Tee) ist vorteilhaft. Durch die Expression eines Gens, das für eine Δ -6-Desaturase aus Moos codiert, in den oben genannten Organismen
35 können Gehalte an ungesättigten Fettsäuren in den Organismen von mindestens 1 Mol-%, bevorzugt mindestens 3 Mol-%, besonders bevorzugt mindestens 4 Mol-%, ganz besonders bevorzugt mindestens 5 Mol-% erreicht werden.

- 40 Unter Derivate(n) sind beispielsweise funktionelle Homologe der von SEQ ID NO: 1 codierten Enzyme oder deren enzymatischer Aktivität, das heißt Enzyme, die dieselben enzymatischen Reaktionen wie die von SEQ ID NO: 1 katalysieren, zu verstehen. Diese Gene ermöglichen ebenfalls eine vorteilhafte Herstellung
45 von ungesättigten Fettsäuren mit Doppelbindungen in $\Delta 6$ -Position. Unter ungesättigten Fettsäuren sind im folgenden doppelt oder mehrfach ungesättigte Fettsäuren, die Doppelbindungen aufweisen,

zu verstehen. Die Doppelbindungen können konjugiert oder nicht konjugiert sein. Die in SEQ ID NO: 1 genannte Sequenz codiert für ein Enzym, das eine $\Delta 6$ -Desaturase-Aktivität aufweist.

- 5 Das erfindungsgemäße Enzym $\Delta 6$ -Desaturase führt vorteilhaft in Fettsäurereste von Glycerolipiden eine *cis*-Doppelbindung in Position C₆-C₇ ein (siehe SEQ ID NO: 1). Das Enzym hat außerdem eine $\Delta 6$ -Desaturase-Aktivität, die vorteilhaft in Fettsäurereste von Glycerolipiden ausschließlich eine *cis*-Doppelbindung in
- 10 Position C₆-C₇ einführt. Diese Aktivität hat auch das Enzym mit der in SEQ ID NO: 1 genannten Sequenz, bei dem es sich um eine monofunktionelle $\Delta 6$ -Desaturase handelt.

- Die im erfindungsgemäßen Verfahren verwendeten Nukleinsäure-
- 15 sequenz(en) (für die Anmeldung soll der singular den plural umfassen und umgekehrt) oder Fragmente davon können vorteilhaft zur Isolierung weiterer genomischer Sequenzen über Homologie-screening verwendet werden.

- 20 Die genannten Derivate lassen sich beispielsweise aus anderen Organismen eukaryontischen Organismen wie Pflanzen wie speziell Moosen, Dinoflagellaten oder Pilze isolieren.

- Weiterhin sind unter Derivaten bzw. funktionellen Derivaten der
- 25 in SEQ ID NO: 1 genannten Sequenz beispielsweise Allelvarianten zu verstehen, die mindestens 50 % Homologie auf der abgeleiteten Aminosäureebene, vorteilhaft mindestens 70 % Homologie, bevorzugt mindestens 80 % Homologie, besonders bevorzugt mindestens 85 % Homologie, ganz besonders bevorzugt 90 % Homologie aufweisen.
- 30 Die Homologie wurde über den gesamten Aminosäurebereich berechnet. Es wurde das Programm PileUp, BESTFIT, GAP, TRANSLATE bzw. BACKTRANSLATE (= Bestandteil des Programmpaketes UWGCG, Wisconsin Package, Version 10.0-UNIX, January 1999, Genetics Computer Group, Inc., Deverux et al., Nucleic Acid Res., 12,
- 35 1984: 387-395) verwendet (J. Mol. Evolution., 25, 351-360, 1987, Higgins et al., CABIOS, 5 1989: 151-153). Die von den genannten Nukleinsäuren abgeleitete Aminosäuresequenz ist Sequenz SEQ ID NO: 2 zu entnehmen. Unter Homologie ist Identität zu verstehen, das heißt die Aminosäuresequenzen sind zu mindestens
- 40 50 % identisch. Die erfindungsgemäßen Sequenzen sind auf Nukleinsäureebene mindestens 65 % homolog, bevorzugt mindestens 70 %, besonders bevorzugt 75 %, ganz besonders bevorzugt mindestens 80 %.

- 45 Allelvarianten umfassen insbesondere funktionelle Varianten, die durch Deletion, Insertion oder Substitution von Nukleotiden aus der in SEQ ID NO: 1 dargestellten Sequenz erhältlich sind, wobei

die enzymatische Aktivität der abgeleiteten synthetisierten Proteine erhalten bleibt.

Solche DNA-Sequenzen lassen sich ausgehend von der in

- 5 SEQ ID NO: 1 beschriebenen DNA-Sequenz oder Teilen dieser Sequenzen, beispielsweise mit üblichen Hybridisierungsverfahren oder der PCR-Technik aus anderen Eukaryonten wie beispielsweise den oben genannt isolieren. Diese DNA-Sequenzen hybridisieren unter Standardbedingungen mit den genannten Sequenzen. Zur
- 10 Hybridisierung werden vorteilhaft kurze Oligonukleotide beispielsweise der konservierten Bereiche, die über Vergleiche mit anderen Desaturasegenen in dem Fachmann bekannter Weise ermittelt werden können, verwendet. Vorteilhaft werden die Histidin-Box-Sequenzen verwendet. Es können aber auch längere Fragmente der
- 15 erfindungsgemäßen Nukleinsäuren oder die vollständigen Sequenzen für die Hybridisierung verwendet werden. Je nach der verwendeten Nukleinsäure: Oligonukleotid, längeres Fragment oder vollständige Sequenz oder je nachdem welche Nukleinsäureart DNA oder RNA für die Hybridisierung verwendet werden, variieren diese Standard-
- 20 bedingungen. So liegen beispielsweise die Schmelztemperaturen für DNA:DNA-Hybride ca. 10°C niedriger als die von DNA:RNA-Hybriden gleicher Länge.

- Unter Standardbedingungen sind beispielsweise je nach Nukleinsäure Temperaturen zwischen 42 und 58°C in einer wäßrigen Pufferlösung mit einer Konzentration zwischen 0,1 bis 5 x SSC (1 x SSC = 0,15 M NaCl, 15 mM Natriumcitrat, pH 7,2) oder zusätzlich in Gegenwart von 50 % Formamid wie beispielsweise 42°C in 5 x SSC, 50 % Formamid zu verstehen. Vorteilhafterweise liegen die
- 25 Hybridisierungsbedingungen für DNA:DNA-Hybride bei 0,1 x SSC und Temperaturen zwischen etwa 20°C bis 45°C, bevorzugt zwischen etwa 30°C bis 45°C. Für DNA:RNA-Hybride liegen die Hybridisierungsbedingungen vorteilhaft bei 0,1 x SSC und Temperaturen zwischen etwa 30°C bis 55°C, bevorzugt zwischen etwa 45°C bis 55°C. Diese
- 30 angegebenen Temperaturen für die Hybridisierung sind beispielhaft kalkulierte Schmelztemperaturwerte für eine Nukleinsäure mit einer Länge von ca. 100 Nukleotiden und einem G + C-Gehalt von 50 % in Abwesenheit von Formamid. Die experimentellen Bedingungen für die DNA-Hybridisierung sind in einschlägigen Lehrbüchern der
- 35 Genetik wie beispielsweise Sambrook et al., "Molecular Cloning", Cold Spring Harbor Laboratory, 1989, beschrieben und lassen sich nach dem Fachmann bekannten Formeln beispielsweise abhängig von der Länge der Nukleinsäuren, der Art der Hybride oder dem G + C-Gehalt berechnen. Weitere Informationen zur Hybridisierung kann
- 40 der Fachmann folgenden Lehrbüchern entnehmen: Ausubel et al. (eds), 1985, Current Protocols in Molecular Biology, John Wiley & Sons, New York; Hames and Higgins (eds), 1985, Nucleic Acids

Hybridization: A Practical Approach, IRL Press at Oxford University Press, Oxford; Brown (ed), 1991, Essential Molecular Biology: A Practical Approach, IRL Press at Oxford University Press, Oxford.

5

Weiterhin sind unter Derivaten Homologe der Sequenz SEQ ID No: 1 beispielsweise eukaryontische Homologe, verkürzte Sequenzen, Einzelstrang-DNA der codierenden und nichtcodierenden DNA-Sequenz oder RNA der codierenden und nichtcodierenden DNA-Sequenz zu

10 verstehen.

Außerdem sind unter Homologen der Sequenz SEQ ID NO: 1 Derivate wie beispielsweise Promotorvarianten zu verstehen. Diese Varianten können durch ein oder mehrere Nukleotidaustausche, durch

15 Insertion(en) und/oder Deletion(en) verändert sein, ohne daß aber die Funktionalität bzw. Wirksamkeit der Promotoren beeinträchtigt sind. Des weiteren können die Promotoren durch Veränderung ihrer Sequenz in ihrer Wirksamkeit erhöht oder komplett durch wirksamere Promotoren auch artfremder Organismen ausgetauscht werden.

20

Unter Derivaten sind auch vorteilhaft Varianten zu verstehen, deren Nukleotidsequenz im Bereich -1 bis -2000 vor dem Startcodon so verändert wurden, daß die Genexpression und/oder die Proteinexpression verändert, bevorzugt erhöht wird. Weiterhin sind unter

25 Derivaten auch Varianten zu verstehen, die am 3'-Ende verändert wurden.

30

Die Nukleinsäuresequenzen, die für eine $\Delta 6$ -Desaturase codiert, können synthetisch hergestellt oder natürlich gewonnen sein oder

30 eine Mischung aus synthetischen und natürlichen DNA-Bestandteilen enthalten, sowie aus verschiedenen heterologen $\Delta 6$ -Desaturase-Genabschnitten verschiedener Organismen bestehen. Im allgemeinen werden synthetische Nukleotid-Sequenzen mit Codons erzeugt, die von den entsprechenden Wirtsorganismen beispielsweise Pflanzen

35 bevorzugt werden. Dies führt in der Regel zu einer optimalen Expression der heterologen Gene. Diese von Pflanzen bevorzugten Codons können aus Codons mit der höchsten Proteinhäufigkeit bestimmt werden, die in den meisten interessanten Pflanzenspezies exprimiert werden. Ein Beispiel für *Corynebacterium glutamicum* ist gegeben in: Wada et al. (1992) *Nucleic Acids Res.* 20:2111-2118). Die Durchführung solcher Experimente sind mit Hilfe von Standardmethoden durchführbar und sind dem Fachmann auf dem Gebiet bekannt.

40

45 Funktionell äquivalente Sequenzen, die für das $\Delta 6$ -Desaturase-Gen codieren, sind solche Derivate der erfindungsgemäßen Sequenz, welche trotz abweichender Nukleotidsequenz noch die gewünschten

Funktionen, das heißt die enzymatische Aktivität der Proteine besitzen. Funktionelle Äquivalente umfassen somit natürlich vorkommende Varianten der hierin beschriebenen Sequenzen sowie künstliche, z.B. durch chemische Synthese erhaltene, an den

5 Codon-Gebrauch einer Pflanze angepaßte, künstliche Nukleotid-Sequenzen.

Außerdem sind artifizielle DNA-Sequenzen geeignet, solange sie, wie oben beschrieben, die gewünschte Eigenschaft beispielsweise

10 weise der Erhöhung des Gehaltes von $\Delta 6$ -Doppelbindungen in Fettsäuren, Ölen oder Lipiden in der Pflanze durch Überexpression des $\Delta 6$ -Desaturase-Gens in Kulturpflanzen vermitteln. Solche artifiziellen DNA-Sequenzen können beispielsweise durch Rückübersetzung mittels Molecular Modelling konstruierter Proteine,

15 die $\Delta 6$ -Desaturase-Aktivität aufweisen oder durch in vitro-Selektion ermittelt werden. Mögliche Techniken zur in vitro-Evolution von DNA zur Veränderung bzw. Verbesserung der DNA-Sequenzen sind beschrieben bei Patten, P.A. et al., Current Opinion in Biotechnology 8, 724-733 (1997) oder bei Moore, J.C.

20 et al., Journal of Molecular Biology 272, 336-347 (1997). Besonders geeignet sind codierende DNA-Sequenzen, die durch Rückübersetzung einer Polypeptidsequenz gemäß der für die Wirtspflanze spezifischen Codon-Nutzung erhalten werden. Die spezifische Codon-Nutzung kann ein mit pflanzen genetischen

25 Methoden vertrauter Fachmann durch Computerauswertungen anderer, bekannter Gene der zu transformierenden Pflanze leicht ermitteln.

Als weitere geeignete äquivalente Nukleinsäure-Sequenzen sind zu nennen Sequenzen, welche für Fusionsproteine codieren, wobei

30 Bestandteil des Fusionsproteins ein $\Delta 6$ -Desaturase-Polypeptid oder ein funktionell äquivalenter Teil davon ist. Der zweite Teil des Fusionsproteins kann z.B. ein weiteres Polypeptid mit enzymatischer Aktivität sein oder eine antigene Polypeptidsequenz mit deren Hilfe ein Nachweis auf $\Delta 6$ -Desaturase-Expression möglich

35 lich ist (z.B. myc-tag oder his-tag). Bevorzugt handelt es sich dabei jedoch um eine regulative Proteinsequenz, wie z.B. ein Signalsequenz für das ER, das das $\Delta 6$ -Desaturase-Protein an den gewünschten Wirkort leitet.

40 Vorteilhaft können die $\Delta 6$ -Desaturase-Gene im erfindungsgemäßen Verfahren mit weiteren Genen der Fettsäurebiosynthese kombiniert werden. Beispiele für derartige Gene sind die Acetyltransferasen, weitere Desaturasen oder Elongasen ungesättigter oder gesättigter Fettsäuren wie in WO 00/12720 beschrieben. Für die in-vivo und

45 speziell in-vitro Synthese ist die Kombination mit z.B. NADH-Cytochrom B5 Reduktasen vorteilhaft, die Reduktionsäquivalente aufnehmen oder abgeben können.

- Unter den im erfindungsgemäßen Verfahren verwendeten Proteine sind Proteine zu verstehen, die eine in der Sequenz SEQ ID NO: 2 dargestellte Aminosäuresequenz oder eine daraus durch Substitution, Inversion, Insertion oder Deletion von einem oder
- 5 mehreren Aminosäureresten erhältliche Sequenz enthalten, wobei die enzymatische Aktivität des in SEQ ID NO: 2 dargestellten Proteins erhalten bleibt bzw. nicht wesentlich reduziert wird. Unter nicht wesentlich reduziert sind alle Enzyme zu verstehen, die noch mindestens 10 %, bevorzugt 20 %, besonders bevorzugt
- 10 30 % der enzymatischen Aktivität des Ausgangsenzyms aufweisen. Dabei können beispielsweise bestimmte Aminosäuren durch solche mit ähnlichen physikochemischen Eigenschaften (Raumerfüllung, Basizität, Hydrophobizität etc.) ersetzt werden. Beispielsweise werden Argininreste gegen Lysinreste, Valinreste gegen Isoleucin-
- 15 reste oder Asparaginsäurereste gegen Glutaminsäurereste ausgetauscht. Es können aber auch ein oder mehrere Aminosäuren in ihrer Reihenfolge vertauscht, hinzugefügt oder entfernt werden, oder es können mehrere dieser Maßnahmen miteinander kombiniert werden.
- 20
- Unter Derivaten sind auch funktionelle Äquivalente zu verstehen, die insbesondere auch natürliche oder künstliche Mutationen einer ursprünglich isolierten für $\Delta 6$ -Desaturase codierende Sequenz beinhalten, welche weiterhin die gewünschte Funktion zeigen, das
- 25 heißt das deren enzymatische Aktivität nicht wesentlich reduziert ist. Mutationen umfassen Substitutionen, Additionen, Deletionen, Vertauschungen oder Insertionen eines oder mehrerer Nukleotidreste. Somit werden beispielsweise auch solche Nukleotidsequenzen durch die vorliegende Erfindung mit umfaßt, welche man durch
- 30 Modifikation der $\Delta 6$ -Desaturase Nukleotidsequenz erhält. Ziel einer solchen Modifikation kann z.B. die weitere Eingrenzung der darin enthaltenen codierenden Sequenz oder z.B. auch die Einfügung weiterer Restriktionsenzym-Schnittstellen sein.
- 35 Funktionelle Äquivalente sind auch solche Varianten, deren Funktion, verglichen mit dem Ausgangsgen bzw. Genfragment, abgeschwächt (= nicht wesentlich reduziert) oder verstärkt ist (= Enzymaktivität ist stärker als die Aktivität des Ausgangsenzym, das heißt Aktivität ist höher als 100 %, bevorzugt höher
- 40 als 110 %, besonders bevorzugt höher als 130 %).

Die im erfindungsgemäßen Verfahren verwendeten oben genannten Nukleinsäuresequenzen werden vorteilhaft zum Einbringen in einen Wirtsorganismus in eine Expressionskassette inseriert.

- 45 Die Nukleinsäuresequenzen können jedoch auch direkt in den Wirtsorganismus eingebracht werden. Die Nukleinsäuresequenz kann dabei vorteilhaft beispielsweise eine DNA- oder cDNA-Sequenz sein.

- Zur Insertion in eine Expressionskassette geeignete codierende Sequenzen sind beispielsweise solche, die für eine $\Delta 6$ -Desaturase mit den oben beschriebenen Sequenzen codieren und die dem Wirt die Fähigkeit zur Überproduktion von Fettsäuren, Ölen oder
- 5 Lipiden mit Doppelbindungen in $\Delta 6$ -Position verleihen. Diese Sequenzen können homologen oder heterologen Ursprungs sein.

- Unter einer Expressionskassette (= Nukleinsäurekonstrukt oder -fragment) ist die in SEQ ID NO: 1 genannte Sequenz, die sich
- 10 als Ergebnis des genetischen Codes und/oder deren funktionellen oder nicht funktionellen Derivate zu verstehen, die mit einem oder mehreren Regulationssignalen vorteilhafterweise zur Erhöhung der Genexpression funktionell verknüpft wurden und welche die Expression der codierenden Sequenz in der Wirtszelle steuern.
- 15 Diese regulatorischen Sequenzen sollen die gezielte Expression der Gene und der Proteinexpression ermöglichen. Dies kann beispielsweise je nach Wirtsorganismus bedeuten, daß das Gen erst nach Induktion exprimiert und/oder überexprimiert wird, oder daß es sofort exprimiert und/oder überexprimiert wird. Beispielsweise
- 20 handelt es sich bei diesen regulatorischen Sequenzen um Sequenzen an die Induktoren oder Repressoren binden und so die Expression der Nukleinsäure regulieren. Zusätzlich zu diesen neuen Regulationssequenzen oder anstelle dieser Sequenzen kann die natürliche Regulation dieser Sequenzen vor den eigentlichen Struktur-
- 25 genen noch vorhanden sein und gegebenenfalls genetisch verändert worden sein, so daß die natürliche Regulation ausgeschaltet und die Expression der Gene erhöht wurde. Das Genkonstrukt kann aber auch einfacher aufgebaut sein, das heißt es wurden keine zusätzlichen Regulationssignale vor die Nukleinsäuresequenz oder dessen
- 30 Derivate inseriert und der natürliche Promotor mit seiner Regulation wurde nicht entfernt. Stattdessen wurde die natürliche Regulationssequenz so mutiert, daß keine Regulation mehr erfolgt und/oder die Genexpression gesteigert wird. Diese veränderten Promotoren können in Form von Teilsequenzen (= Promotor mit
- 35 Teilen der erfindungsgemäßen Nukleinsäuresequenzen) auch allein vor das natürliche Gen zur Steigerung der Aktivität gebracht werden. Das Genkonstrukt kann außerdem vorteilhafterweise auch eine oder mehrere sogenannte "enhancer Sequenzen" funktionell verknüpft mit dem Promotor enthalten, die eine erhöhte Expression
- 40 der Nukleinsäuresequenz ermöglichen. Auch am 3'-Ende der DNA-Sequenzen können zusätzliche vorteilhafte Sequenzen inseriert werden wie weitere regulatorische Elemente oder Terminatoren. Das $\Delta 6$ -Desaturase-Gen kann in einer oder mehreren Kopien in der Expressionskassette (= Genkonstrukt) enthalten sein. Auch
- 45 eventuell mit exprimierte Gene, die vorteilhaft an der Fettsäurebiosynthese beteiligt sind, können in einer oder mehreren Kopien in der Expressionskassette vorhanden sein.

13

- Die regulatorischen Sequenzen bzw. Faktoren können dabei wie oben beschrieben vorzugsweise die Genexpression der eingeführten Gene positiv beeinflussen und dadurch erhöhen. So kann eine Verstärkung der regulatorischen Elemente vorteilhafterweise auf der
- 5 Transkriptionsebene erfolgen, indem starke Transkriptionssignale wie Promotoren und/oder "Enhancer" verwendet werden. Daneben ist aber auch eine Verstärkung der Translation möglich, indem beispielsweise die Stabilität der mRNA verbessert wird.
- 10 Als Promotoren in der Expressionskassette sind grundsätzlich alle Promotoren geeignet, die die Expression von Fremdgenen in Organismen vorteilhaft in Pflanzen oder Pilzen steuern können. Vorzugsweise verwendet man insbesondere einen pflanzlichen Promotor oder Promotoren, die beispielsweise aus einem Pflanzen-
- 15 virus entstammen. Vorteilhafte Regulationssequenzen für das erfindungsgemäße Verfahren sind beispielsweise in Promotoren wie *cos-*, *tac-*, *trp-*, *tet-*, *trp-tet-*, *lpp-*, *lac-*, *lpp-lac-*, *lacI^q-*, *T7-*, *T5-*, *T3-*, *gal-*, *trc-*, *ara-*, *SP6-*, λ -P_R- oder im λ -P_L-Promotor enthalten, die vorteilhafterweise in gram-negativen Bakterien
- 20 Anwendung finden. Weitere vorteilhafte Regulationssequenzen sind beispielsweise in den gram-positiven Promotoren *amy* und *SPO2*, in den Hefe- oder Pilzpromotoren *ADC1*, *MF α* , *AC*, *P-60*, *CYC1*, *GAPDH*, *TEF*, *rp28*, *ADH* oder in den Pflanzenpromotoren wie *CaMV/35S* [Franck et al., Cell 21(1980) 285-294], *RUBISCO SSU*, *OCS*, *B33*,
- 25 *nos* (= Nopalinsynthase Promotor) oder im Ubiquitin-Promotor enthalten. Die Expressionskassette kann auch einen chemisch induzierbaren Promotor enthalten, durch den die Expression des exogenen $\Delta 6$ -Desaturase-Gens in den Organismen vorteilhaft in den Pflanzen zu einem bestimmten Zeitpunkt gesteuert werden kann.
- 30 Derartige vorteilhafte Pflanzenpromotoren sind beispielsweise der *PRP1*-Promotor [Ward et al., Plant. Mol. Biol. 22 (1993), 361-366], ein durch Benzensulfonamid-induzierbarer (EP 388186), ein durch Tetrazyklin-induzierbarer (Gatz et al., (1992) Plant J. 2,397-404), ein durch Salizylsäure induzierbarer Promotor
- 35 (WO 95/19443), ein durch Abscisinsäure-induzierbarer (EP335528) bzw. ein durch Ethanol- oder Cyclohexanon-induzierbarer (WO 93/21334) Promotor. Weitere Pflanzenpromotoren sind beispielsweise der Promotor der cytosolischen FBPase aus Kartoffel, der *ST-LSI* Promotor aus Kartoffel (Stockhaus et al., EMBO J.
- 40 8 (1989) 2445-245), der Promotor der Phosphoribosylpyrophosphat Amidotransferase aus *Glycine max* (siehe auch Genbank Accession Nummer U87999) oder ein Nodien-spezifischen Promotor wie in EP 249676 können vorteilhaft verwandt werden. Vorteilhaft sind insbesondere solche pflanzliche Promotoren, die die Expression in
- 45 Geweben oder Pflanzenteilen/-organen sicherstellen, in denen die Fettsäurebiosynthese bzw. dessen Vorstufen stattfindet wie beispielsweise im Endosperm oder im sich entwickelnden Embryo. Ins-

besondere zu nennen sind vorteilhafte Promotoren, die eine samen-spezifische Expression gewährleisten wie beispielsweise der USP-Promotor oder Derivate davon, der LEB4-Promotor, der Phaseolin-Promotor oder der Napin-Promotor. Der erfindungsgemäß aufgeführte
5 und besonders vorteilhafte USP-Promotor oder dessen Derivate vermitteln in der Samenentwicklung eine sehr früh Genexpression (Baeumlein et al., Mol Gen Genet, 1991, 225 (3): 459-67). Weitere vorteilhafte samenspezifische Promotoren, die für monokotyle und dikotyle Pflanzen verwendet werden können, sind die für Dikotyle
10 geeignete Promotoren wie ebenfalls beispielhaft ausgeführte Napingen-Promotor aus Raps (US5,608,152), der Oleosin-Promotor aus Arabidopsis (WO98/45461), der Phaseolin-Promotor aus Phaseolus vulgaris (US5,504,200), der Bce4-Promotor aus Brassica (WO91/13980) oder der Leguminosen B4-Promotor (LeB4, Baeumlein
15 et al., Plant J., 2, 2, 1992: 233 - 239) oder für Monokotyle geeignete Promotoren wie die Promotoren des lpt2- oder lpt1-Gens aus Gerste (WO95/15389 und WO95/23230) oder die Promotoren des Gersten Hordein-Gens, des Reis Glutelin-Gens, des Reis Oryzin-Gens, des Reis Prolamin-Gens, des Weizen Gliadin-
20 Gens, des Weizen Glutelin-Gens, des Mais Zein-Gens, des Hafer Glutelin-Gens, des Sorghum Kasirin-Gens oder des Roggen Secalin-Gens, die in WO99/16890 beschrieben werden.

Weiterhin sind insbesondere solche Promotoren bevorzugt, die
25 die Expression in Geweben oder Pflanzenteilen sicherstellen, in denen beispielsweise die Biosynthese von Fettsäuren, Ölen und Lipiden bzw. deren Vorstufen stattfindet. Insbesondere zu nennen sind Promotoren, die eine samenspezifische Expression gewährleisten. Zu nennen sind der Promotor des Napin-Gens aus Raps
30 (US 5,608,152), des USP-Promotor aus Vicia faba (USP=unbekanntes Samenprotein, Baeumlein et al., Mol Gen Genet, 1991, 225 (3): 459-67), des Oleosin-Gens aus Arabidopsis (WO98/45461), des Phaseolin-Promotors (US 5,504,200) oder der Promotor des Legumin B4-Gens (LeB4; Baeumlein et al., 1992, Plant Journal, 2 (2):
35 233-9). Weiterhin sind zu nennen Promotoren, wie der des lpt2 oder lpt1-Gens aus Gerste (WO95/15389 und WO95/23230), die in monokotylen Pflanzen samenspezifische Expression vermitteln.

In der Expressionskassette (= Genkonstrukt, Nukleinsäurekon-
40 strukt) können wie oben beschrieben noch weitere Gene, die in die Organismen eingebracht werden sollen, enthalten sein. Diese Gene können unter getrennter Regulation oder unter der gleichen Regulationsregion wie das $\Delta 6$ -Desaturase-Gen liegen. Bei diesen Genen handelt es sich beispielsweise um weitere Biosynthesegene
45 vorteilhaft der Fettsäurebiosynthese, die eine gesteigerte Synthese ermöglichen. Beispielsweise seien die Gene für die $\Delta 15$ -, $\Delta 12$ -, $\Delta 9$ -, $\Delta 5$ -, $\Delta 4$ -Desaturase, die verschiedenen Hydroxylasen,

die Acyl-ACP-Thioesterasen, β -Ketoacyl-Synthasen oder β -Ketoacyl-Reductasen genannt. Vorteilhaft werden die Desaturasegene im Nukleinsäurekonstrukt verwendet.

5 Prinzipiell können alle natürlichen Promotoren mit ihren Regulationssequenzen wie die oben genannten für die erfindungsgemäße Expressionskassette und das erfindungsgemäße Verfahren, wie unten beschrieben, verwendet werden. Darüberhinaus können auch synthetische Promotoren vorteilhaft verwendet werden.

10

Es können verschiedene DNA-Fragmente manipuliert werden, um eine Nukleotid-Sequenz zu erhalten, die zweckmäßigerweise in der korrekten Richtung gelesen wird und die mit einem korrekten Leseraster ausgestattet ist. Für die Verbindung der DNA-Fragmente

15 (= erfindungsgemäße Nukleinsäuren) miteinander können an die Fragmente Adaptoren oder Linker angesetzt werden.

Zweckmäßigerweise können die Promotor- und die Terminator-Regionen in Transkriptionsrichtung mit einem Linker oder Poly-

20 linker, der eine oder mehrere Restriktionsstellen für die Insertion dieser Sequenz enthält, versehen werden. In der Regel hat der Linker 1 bis 10, meistens 1 bis 8, vorzugsweise 2 bis 6 Restriktionsstellen. Im allgemeinen hat der Linker innerhalb der regulatorischen Bereiche eine Größe von weniger als 100 bp, 25 häufig weniger als 60 bp, mindestens jedoch 5 bp. Der Promotor kann sowohl nativ bzw. homolog als auch fremdartig bzw. heterolog zum Wirtsorganismus beispielsweise zur Wirtspflanze sein. Die Expressionskassette beinhaltet in der 5'-3'-Transkriptionsrichtung den Promotor, eine DNA-Sequenz, die für ein im er- 30 findungsgemäßen Verfahren verwendetes $\Delta 6$ -Desaturase-Gen codiert und eine Region für die transkriptionale Termination. Verschiedene Terminationsbereiche sind gegeneinander beliebig austauschbar.

35 Ferner können Manipulationen, die passende Restriktionsschnittstellen bereitstellen oder die überflüssige DNA oder Restriktionsschnittstellen entfernen, eingesetzt werden. Wo Insertionen, Deletionen oder Substitutionen wie z.B. Transitionen und Transversionen in Frage kommen, können *in vitro*-Mutagenese, -primer-repair-, Restriktion oder Ligation verwendet werden. Bei geeigneten Manipulationen, wie z.B. Restriktion, -chewing-back- oder Auffüllen von Überhängen für -bluntends-, können komplementäre Enden der Fragmente für die Ligation zur Verfügung gestellt werden.

45

Von Bedeutung für eine vorteilhafte hohe Expression kann u.a. das Anhängen des spezifischen ER-Retentionssignals SEKDEL sein (Schouten, A. et al., Plant Mol. Biol. 30 (1996), 781-792), die durchschnittliche Expressionshöhe wird damit verdreifacht bis
5 vervierfacht. Es können auch andere Retentionssignale, die natürlicherweise bei im ER lokalisierten pflanzlichen und tierischen Proteinen vorkommen, für den Aufbau der Kassette eingesetzt werden.

- 10 Bevorzugte Polyadenylierungssignale sind pflanzliche Polyadenylierungssignale, vorzugsweise solche, die im wesentlichen T-DNA-Polyadenylierungssignale aus *Agrobacterium tumefaciens*, insbesondere des Gens 3 der T-DNA (Octopin Synthase) des Ti-Plasmids pTiACH5 entsprechen (Gielen et al., EMBO J. 3 (1984),
15 835 ff) oder entsprechende funktionelle Äquivalente.

Die Herstellung einer Expressionskassette erfolgt durch Fusion eines geeigneten Promotors mit einer geeigneten $\Delta 6$ -Desaturase-DNA-Sequenz sowie einem Polyadenylierungssignal nach gängigen

- 20 Rekombinations- und Klonierungstechniken, wie sie beispielsweise in T. Maniatis, E.F. Fritsch und J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989) sowie in T.J. Silhavy, M.L. Berman und L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor
25 Laboratory, Cold Spring Harbor, NY (1984) und in Ausubel, F.M. et al., Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley-Interscience (1987) beschrieben werden.

Die DNA Sequenz codierend für eine $\Delta 6$ -Desaturase aus *Phyco-*

- 30 *mitrella patens* beinhaltet alle Sequenzmerkmale, die notwendig sind, um eine dem Ort der Fettsäure-, Lipid- oder Ölbiosynthese korrekte Lokalisation zu erreichen. Daher sind keine weiteren Targetingsequenzen per se notwendig. Allerdings kann eine solche Lokalisation wünschenswert und vorteilhaft sein und daher künstlich verändert oder verstärkt werden, sodaß auch solche Fusionskonstrukte eine bevorzugte vorteilhafte Ausführungsform der Erfindung sind.

Insbesondere bevorzugt sind Sequenzen, die ein Targeting in

- 40 Plastiden gewährleisten. Unter bestimmten Umständen kann auch ein Targeting in andere Kompartimente (referiert: Kermode, Crit. Rev. Plant Sci. 15, 4 (1996), 285-423) z.B. in die Vakuole, in das Mitochondrium, in das Endoplasmatische Retikulum (ER), Peroxisomen, Lipidkörper oder durch ein Fehlen entsprechender
45 operativer Sequenzen ein Verbleib im Kompartiment des Entstehens, dem Zytosol, wünschenswert sein.

Vorteilhafterweise werden die für $\Delta 6$ -Desaturase-Gene codierenden Nukleinsäuresequenzen zusammen mit mindestens einem Reporter-Gen in eine Expressionskassette kloniert, die in den Organismus über einen Vektor oder direkt in das Genom eingebracht wird. Dieses Reporter-Gen sollte eine leichte Detektierbarkeit über einen Wachstums-, Fluoreszenz-, Chemo-, Biolumineszenz- oder Resistenz-assay oder über eine photometrische Messung ermöglichen. Beispielsweise seien als Reporter-Gene Antibiotika- oder Herbizid-resistenzgene, Hydrolasegene, Fluoreszenzproteingene, Biolumineszenzgene, Zucker- oder Nukleotidstoffwechselgene oder Biosynthesegene wie das Ura3-Gen, das Ilv2-Gen, das Luciferasegen, das β -Galactosidasegen, das gfp-Gen, das 2-Desoxyglucose-6-phosphat-Phosphatasegen, das β -Glucuronidase-Gen, β -Lactamasegen, das Neomycinphosphotransferasegen, das Hygromycinphosphotransferasegen oder das BASTA (= Gluphosinatresistenz)-Gen genannt. Diese Gene ermöglichen eine leichte Meßbarkeit und Quantifizierbarkeit der Transkriptionsaktivität und damit der Expression der Gene. Damit lassen sich Genomstellen identifizieren, die eine unterschiedliche Produktivität zeigen.

Gemäß einer bevorzugten Ausführungsform umfaßt eine Expressionskassette stromaufwärts, d.h. am 5'-Ende der codierenden Sequenz, einen Promotor und stromabwärts, d.h. am 3'-Ende, ein Polyadenylierungssignal und gegebenenfalls weitere regulatorische Elemente, welche mit der dazwischenliegenden codierenden Sequenz für die $\Delta 6$ -Desaturase DNA Sequenz operativ verknüpft sind. Unter einer operativen Verknüpfung versteht man die sequenzielle Anordnung von Promotor, codierender Sequenz, Terminator und ggf. weiterer regulatorischer Elemente derart, daß jedes der regulatorischen Elemente seine Funktion bei der Expression der codierenden Sequenz bestimmungsgemäß erfüllen kann. Die zur operativen Verknüpfung bevorzugten Sequenzen sind Targeting-Sequenzen zur Gewährleistung der subzellulären Lokalisation in Plastiden. Aber auch Targeting-Sequenzen zur Gewährleistung der subzellulären Lokalisation im Mitochondrium, im Endoplasmatischen Retikulum (= ER), im Zellkern, in Ölkörperchen oder anderen Kompartimenten sind bei Bedarf einsetzbar sowie Translationsverstärker wie die 5'-Führungssequenz aus dem Tabak-Mosaik-Virus (Gallie et al., Nucl. Acids Res. 15 (1987), 8693-8711).

Eine Expressionskassette kann beispielsweise einen konstitutiven Promotor (bevorzugt den USP- oder Napin-Promotor), das zu exprimierende Gen und das ER-Retentionssignal enthalten. Als ER-Retentionssignal wird bevorzugt die Aminosäuresequenz KDEL (Lysin, Asparaginsäure, Glutaminsäure, Leucin) verwendet.

Die Expressionskassette wird zur Expression in einem prokaryontischen oder eukaryontischen Wirtsorganismus beispielsweise einem Mikroorganismus wie einem Pilz oder einer Pflanze vorteilhafterweise in einen Vektor wie beispielsweise einem Plasmid, einem Phagen oder sonstiger DNA inseriert, der eine optimale Expression der Gene im Wirtsorganismus ermöglicht. Geeignete Plasmide sind beispielsweise in *E. coli* pLG338, pACYC184, pBR-Serie wie z.B. pBR322, pUC-Serie wie pUC18 oder pUC19, M13mp-Serie, pKC30, pRep4, pHS1, pHS2, pPLc236, pMBL24, pLG200, pUR290, pIN-III¹¹³-B1, λ gt11 oder pBdCI, in *Streptomyces* pIJ101, pIJ364, pIJ702 oder pIJ361, in *Bacillus* pUB110, pC194 oder pBD214, in *Corynebacterium* pSA77 oder pAJ667, in Pilzen pALS1, pIL2 oder pBB116, weitere vorteilhafte Pilzvektoren werden von Romanos, M.A. et al., [(1992) "Foreign gene expression in yeast: a review", *Yeast* 8: 423-488] und von van den Hondel, C.A.M.J.J. et al. [(1991) "Heterologous gene expression in filamentous fungi" sowie in *More Gene Manipulations in Fungi* [J.W. Bennet & L.L. Lasure, eds., p. 396-428: Academic Press: San Diego] und in "Gene transfer systems and vector development for filamentous fungi" [van den Hondel, C.A.M.J.J. & Punt, P.J. (1991) in: *Applied Molecular Genetics of Fungi*, Peberdy, J.F. et al., eds., p. 1-28, Cambridge University Press: Cambridge] beschrieben. Vorteilhafte Hefektoren sind beispielsweise 2 μ M, pAG-1, YEpl6, YEpl3 oder pEMBLYe23. Beispiele für Algen- oder Pflanzenpromotoren sind pLGV23, pGHLac⁺, pBIN19, pAK2004, pVKH oder pDH51 (siehe Schmidt, R. and Willmitzer, L., 1988). Die oben genannten Vektoren oder Derivate der vorstehend genannten Vektoren stellen eine kleine Auswahl der möglichen Plasmide dar. Weitere Plasmide sind dem Fachmann wohl bekannt und können beispielsweise aus dem Buch *Cloning Vectors* (Eds. Pouwels P.H. et al. Elsevier, Amsterdam-New York-Oxford, 1985, ISBN 0 444 904018) entnommen werden. Geeignete pflanzliche Vektoren werden unter anderem in "Methods in Plant Molecular Biology and Biotechnology" (CRC Press), Kap. 6/7, S.71-119 beschrieben. Vorteilhafte Vektoren sind sog. shuttle-Vektoren oder binäre Vektoren, die in *E. coli* und *Agrobacterium* replizieren.

Unter Vektoren sind außer Plasmiden auch alle anderen dem Fachmann bekannten Vektoren wie beispielsweise Phagen, Viren wie SV40, CMV, Baculovirus, Adenovirus, Transposons, IS-Elemente, Phasmide, Phagemide, Cosmide, lineare oder zirkuläre DNA zu verstehen. Diese Vektoren können autonom im Wirtsorganismus repliziert oder chromosomal repliziert werden, bevorzugt ist eine chromosomale Replikation.

In einer weiteren Ausgestaltungsform des Vektors kann die erfindungsgemäße Expressionskassette auch vorteilhafterweise in Form einer linearen DNA in die Organismen eingeführt werden und über heterologe oder homologe Rekombination in das Genom des Wirtsorganismus integriert werden. Diese lineare DNA kann aus einem linearisierten Plasmid oder nur aus der Expressionskassette als Vektor oder den erfindungsgemäßen Nukleinsäuresequenzen bestehen.

10 In einer weiteren vorteilhaften Ausführungsform kann die erfindungsgemäße Nukleinsäuresequenz auch alleine in einen Organismus eingebracht werden.

Sollen neben der erfindungsgemäßen Nukleinsäuresequenz weitere Gene in den Organismus eingeführt werden, so können alle zusammen mit einem Reportergen in einem einzigen Vektor oder jedes einzelne Gen mit einem Reportergen in je einem Vektor oder mehrere Gene zusammen in verschiedenen Vektoren in den Organismus eingebracht werden, wobei die verschiedenen Vektoren gleichzeitig oder sukzessive eingebracht werden können.

Der Vektor enthält vorteilhaft mindestens eine Kopie der Nukleinsäuresequenzen, die für eine $\Delta 6$ -Desaturase codieren, und/oder der Expressionskassette.

25 Beispielhaft kann die pflanzliche Expressionskassette in den Transformationsvektor pRT ((a) Toepfer et al., 1993, Methods Enzymol., 217: 66-78; (b) Toepfer et al. 1987, Nucl. Acids. Res. 15: 5890 ff.) eingebaut werden.

30 Alternativ kann ein rekombinanter Vektor (= Expressionsvektor) auch in-vitro transkribiert und translatiert werden, z.B. durch Nutzung des T7 Promotors und der T7 RNA Polymerase.

35 In Prokaryoten verwendete Expressionsvektoren nutzen häufig induzierbare Systeme mit und ohne Fusionsproteinen bzw Fusions-oligopeptiden, wobei diese Fusionen sowohl N-terminal als auch C-terminal oder anderen nutzbaren Domänen eines Proteins erfolgen können. Solche Fusionsvektoren dienen in der Regel dazu: i.) die Expressionsrate der RNA zu erhöhen ii.) die erzielbare Proteinsyntheserate zu erhöhen, iii.) die Löslichkeit des Proteins zu erhöhen, iv.) oder die Reinigung durch einen für die Affinitätschromatographie nutzbare Bindesequenz zu vereinfachen. Häufig werden auch proteolytische Spaltstellen über Fusionsproteine eingeführt, was die Abspaltung eines Teils des Fusionsproteins auch der Reinigung ermöglicht. Solche Erkennungssequenzen für

Proteasen erkennen sind z.B. Faktor Xa, Thrombin und Entero-kinase.

Typische vorteilhafte Fusions- und Expressionsvektoren sind pGEX
5 [Pharmacia Biotech Inc; Smith, D.B. and Johnson, K.S. (1988) *Gene* 67: 31-40], pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) welches Glutathion S-transferase beinhaltet (GST), Maltose Bindeprotein, oder Protein A.

10 Weitere Beispiele für E. coli Expressionsvektoren sind pTrc [Amann et al., (1988) *Gene* 69:301-315] und pET Vektoren [Studier et al., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 60-89; Stratagene, Amsterdam, Niederlande].

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Weitere vorteilhafte Vektoren zur Verwendung in Hefe sind pYepSec1 (Baldari, et al., (1987) *Embo J.* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933-943), pJRY88 (Schultz et al., (1987) *Gene* 54:113-123), and pYES-Derivate (Invitrogen

20 Corporation, San Diego, CA). Vektoren für die Nutzung in filamentösen Pilzen sind beschrieben in: van den Hondel, C.A.M.J.J. & Punt, P.J. (1991) "Gene transfer systems and vector development for filamentous fungi, in: *Applied Molecular Genetics of Fungi*, J.F. Peberdy, et al., eds., p. 1-28, Cambridge University Press:

25 Cambridge.

Alternativ können auch vorteilhaft Insektenzellexpressionsvektoren genutzt werden z.B. für die Expression in Sf 9 Zellen. Dies sind z.B. die Vektoren der pAc Serie (Smith et al. (1983) *Mol.*

30 *Cell Biol.* 3:2156-2165) und der pVL series (Lucklow and Summers (1989) *Virology* 170:31-39).

Des weiteren können zur Genexpression vorteilhaft Pflanzenzellen oder Algenzellen genutzt werden. Beispiele für Pflanzen-

35 expressionsvektoren finden sich in Becker, D., et al. (1992)

"New plant binary vectors with selectable markers located proximal to the left border", *Plant Mol. Biol.* 20: 1195-1197 oder in Bevan, M.W. (1984) "Binary *Agrobacterium* vectors for plant transformation", *Nucl. Acid. Res.* 12: 8711-8721.

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Weiterhin können die für die $\Delta 6$ -Desaturase codierenden Nukleinsäuresequenzen in Säugerzellen exprimiert werden. Beispiel für entsprechende Expressionsvektoren sind pCDM8 und pMT2PC genannt in: Seed, B. (1987) *Nature* 329:840 oder Kaufman et al.

45 (1987) *EMBO J.* 6: 187-195). Dabei sind vorzugsweise zu nutzende Promotoren viralen Ursprungs wie z.B. Promotoren des Polyoma, Adenovirus 2, Cytomegalovirus oder Simian Virus 40. Weitere

prokaryotische und eukaryotische Expressionssysteme sind genannt in Kapitel 16 und 17 in Sambrook et al., *Molecular Cloning: A Laboratory Manual*. 2nd, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 5 1989.

Das Einbringen der erfindungsgemäßen Nukleinsäuren, der Expressionskassette oder des Vektors in Organismen beispielsweise in Pflanzen kann prinzipiell nach allen dem Fachmann 10 bekannten Methoden erfolgen.

Für Mikroorganismen kann der Fachmann entsprechende Methoden den Lehrbüchern von Sambrook, J. et al. (1989) *Molecular cloning: A laboratory manual*, Cold Spring Harbor Laboratory Press, von 15 F.M. Ausubel et al. (1994) *Current protocols in molecular biology*, John Wiley and Sons, von D.M. Glover et al., *DNA Cloning Vol.1*, (1995), IRL Press (ISBN 019-963476-9), von Kaiser et al. (1994) *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory Press oder Guthrie et al. *Guide to Yeast Genetics and Molecular 20 Biology*, *Methods in Enzymology*, 1994, Academic Press entnehmen.

Die Übertragung von Fremdgenen in das Genom einer Pflanze wird als Transformation bezeichnet. Es werden dabei die beschriebenen Methoden zur Transformation und Regeneration von Pflanzen aus 25 Pflanzengewebe oder Pflanzenzellen zur transienten oder stabilen Transformation genutzt. Geeignete Methoden sind die Protoplastentransformation durch Polyethylenglykol-induzierte DNA-Aufnahme, das biolistische Verfahren mit der Genkanone - die sogenannte particle bombardment Methode -, die Elektroporation, die Inku- 30 bation trockener Embryonen in DNA-haltiger Lösung, die Mikroinjektion und der durch *Agrobacterium* vermittelte Gentransfer. Die genannten Verfahren sind beispielsweise in B. Jenes et al., *Techniques for Gene Transfer*, in: *Transgenic Plants*, Vol. 1, Engineering and Utilization, herausgegeben von S.D. Kung und R. 35 Wu, Academic Press (1993) 128-143 sowie in Potrykus *Annu. Rev. Plant Physiol. Plant Molec. Biol.* 42 (1991) 205-225 beschrieben. Vorzugsweise wird das zu exprimierende Konstrukt in einen Vektor kloniert, der geeignet ist, *Agrobacterium tumefaciens* zu transformieren, beispielsweise pBin19 (Bevan et al., *Nucl. Acids Res.* 40 12 (1984) 8711). Mit einem solchen Vektor transformierte *Agrobakterien* können dann in bekannter Weise zur Transformation von Pflanzen, insbesondere von Kulturpflanzen, wie z.B. von Tabakpflanzen, verwendet werden, indem beispielsweise verwundete Blätter oder Blattstücke in einer *Agrobakterienlösung* gebadet 45 und anschließend in geeigneten Medien kultiviert werden. Die Transformation von Pflanzen mit *Agrobacterium tumefaciens* wird beispielsweise von Höfgen und Willmitzer in *Nucl. Acid Res.*

(1988) 16, 9877 beschrieben oder ist unter anderem bekannt aus F.F. White, Vectors for Gene Transfer in Higher Plants; in Transgenic Plants, Vol. 1, Engineering and Utilization, herausgegeben von S.D. Kung und R. Wu, Academic Press, 1993, S. 15-38.

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Mit einem wie oben beschriebenen Expressionsvektor transformierte Agrobakterien können ebenfalls in bekannter Weise zur Transformation von Pflanzen wie Testpflanzen wie Arabidopsis oder Kulturpflanzen wie Getreide, Mais, Hafer, Roggen, Gerste, Weizen,

- 10 Soja, Reis, Baumwolle, Zuckerrübe, Canola, Triticale, Reis, Sonnenblume, Flachs, Hanf, Kartoffel, Tabak, Tomate, Kaffee, Kakao, Tee, Karotte, Paprika, Raps, Tapioka, Maniok, Pfeilwurz, Tagetes, Alfalfa, Salat und den verschiedenen Baum-, Nuß- und Weinspezies, insbesondere von Öl-haltigen Kulturpflanzen,
- 15 wie Soja, Erdnuß, Rizinus, Borretsch, Lein, Sonnenblume, Canola, Baumwolle, Flachs, Raps, Kokosnuß, Ölpalme, Färbersaflor (*Carthamus tinctorius*) oder Kakaobohne verwendet werden, z.B. indem verwundete Blätter oder Blattstücke in einer Agrobakterienlösung gebadet und anschließend in geeigneten Medien kultiviert
- 20 werden.

Die genetisch veränderten Pflanzenzellen können über alle dem Fachmann bekannten Methoden regeneriert werden. Entsprechende Methoden können den oben genannten Schriften von S.D. Kung und

- 25 R. Wu, Potrykus oder Höfgen und Willmitzer entnommen werden.

Als Organismen bzw. Wirtsorganismen für die erfindungsgemäßen Verfahren verwendeten Nukleinsäuren, die verwendete Expressionskassette oder den verwendeten Vektor eignen sich prinzipiell

- 30 vorteilhaft alle Organismen, die in der Lage sind Fettsäuren speziell ungesättigte Fettsäuren zu synthetisieren bzw. für die Expression rekombinanter Gene geeignet sind. Beispielhaft seien Pflanzen wie Arabidopsis, Asteraceae wie Calendula oder Kulturpflanzen wie Soja, Erdnuß, Rizinus, Sonnenblume, Mais, Baumwolle, Flachs, Raps, Kokosnuß, Ölpalme, Färbersaflor (*Carthamus tinctorius*) oder Kakaobohne, Mikroorganismen wie Pilze beispielsweise die Gattung *Mortierella*, *Saprolegnia* oder *Pythium*, Bakterien wie die Gattung *Escherichia*, Cyanobakterien, Ciliaten, Thrausto- oder Schizichytien, Algen oder Protozoen wie Dino-
- 40 flagellaten wie *Cryptocodium* genannt. Bevorzugt werden Organismen, die natürlicherweise Öle in größeren Mengen synthetisieren können wie Pilze der Gattungen *Mortierella* oder *Pythium* wie *Mortierella alpina*, *Pythium insidiosum* oder Pflanzen wie Soja, Raps, Kokosnuß, Ölpalme, Färbersaflor, Rizinus,
- 45 Calendula, Erdnuß, Kakaobohne oder Sonnenblume, besonders bevorzugt werden Soja, Raps, Sonnenblume, Rizinus, *Mortierella* oder

Pythium. Prinzipiell sind als Wirtsorganismen auch transgene Tiere geeignet beispielsweise *C. elegans*.

Nutzbare Wirtszellen sind weiterhin genannt in: Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990).

Verwendbare Expressionsstämme z.B. solche, die eine geringere Proteaseaktivität aufweisen sind beschrieben in: Gottesman, S., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 119-128.

Dabei kann je nach Wahl des Promotors die Expression des $\Delta 6$ -Desaturase-Gens spezifisch in den Blättern, in den Samen, den Knollen oder anderen Teilen der Pflanze erfolgen. Solche Fettsäuren, Öle oder Lipide mit $\Delta 6$ -Doppelbindungen überproduzierenden transgenen Pflanzen, deren Vermehrungsgut, sowie deren Pflanzenzellen, -gewebe oder -teile, sind ein weiterer Gegenstand der vorliegenden Erfindung. Ein bevorzugter erfindungsgemäßer Gegenstand sind transgene Pflanzen beispielsweise Kulturpflanzen wie Mais, Hafer, Roggen, Weizen, Gerste, Reis, Soja, Zuckerrübe, Canola, Triticale, Sonnenblume, Flachs, Hanf, Tabak, Tomate, Kaffee, Kakao, Tee, Karotte, Paprika, Raps, Tapioka, Maniok, Pfeilwurz, Tagetes, Alfalfa, Salat und den verschiedenen Baum-, Nuß- und Weinspezies, Kartoffel, insbesondere Öl-haltige Kulturpflanzen, wie Soja, Erdnuß, Rizinus, Borretsch, Lein, Sonnenblume, Canola, Baumwolle, Flachs, Raps, Kokosnuß, Ölpalme, Färbersaflor (*Carthamus tinctorius*) oder Kakaobohne, Testpflanzen wie *Arabidopsis* oder sonstige Pflanzen wie Moose oder Algen enthaltend eine erfindungsgemäße funktionelle Nukleinsäuresequenz oder eine funktionelle Expressionskassette. Funktionell bedeutet hierbei, daß ein enzymatisch aktives Enzym gebildet wird.

Die Expressionskassette oder die erfindungsgemäßen Nukleinsäuresequenzen enthaltend eine $\Delta 6$ -Desaturasegenesequenz kann darüber hinaus auch zur Transformation der oben beispielhaft genannten Organismen wie Bakterien, Cyanobakterien, filamentösen Pilzen, Ciliaten, Tiere oder Algen mit dem Ziel einer Erhöhung des Gehaltes an Fettsäuren, Ölen oder Lipiden $\Delta 6$ -Doppelbindungen eingesetzt werden. Bevorzugte transgene Organismen sind Bakterien, Cyanobakterien, filamentöse Pilze oder Algen.

Unter transgenen Organismen sind Organismen zu verstehen, die eine Fremde aus einem anderen Organismus stammende Nukleinsäure, die für eine im erfindungsgemäßen Verfahren verwendete $\Delta 6$ -Desaturase codiert, enthalten. Unter transgenen Organismen sind auch Organismen zu verstehen, die eine Nukleinsäure, die

- aus demselben Organismus stammt und die für eine $\Delta 6$ -Desaturase codiert, enthält, wobei diese Nukleinsäure als zusätzliche Genkopie enthalten ist oder nicht in der natürlichen Nukleinsäureumgebung des $\Delta 6$ -Desaturase-Gens enthalten ist.
- 5 Organismen sind auch Organismen bei denen die natürliche 3'- und/oder 5'-Region des $\Delta 6$ -Desaturase-Gens durch gezielte gentechnologische Veränderungen gegenüber dem Ausgangsorganismus verändert wurde. Bevorzugt sind transgene Organismen bei denen eine Fremd-DNA eingebracht wurde. Besonders bevorzugt sind trans-
- 10 gene Pflanzen, in die Fremd-DNA eingebracht wurde. Unter transgenen Pflanzen sind einzelne Pflanzenzellen und deren Kulturen wie beispielsweise Kalluskulturen auf Festmedien oder in Flüssigkultur, Pflanzenteile und ganze Pflanzen zu verstehen.
- 15 Ein weiterer Erfindungsgegenstand sind transgene Organismen ausgewählt aus der Gruppe Pflanzen, Pilze, Ciliaten, Algen, Bakterien, Cyanobakterien oder Tiere, bevorzugt transgene Pflanzen oder Algen, die mindestens eine isolierte Nukleinsäuresequenz enthalten, die für ein Polypeptid mit $\Delta 6$ -Desaturase-
- 20 aktivität codiert, ausgewählt aus der Gruppe:
- a) einer Nukleinsäuresequenz mit der in SEQ ID NO: 1 dargestellten Sequenz,
 - 25 b) Nukleinsäuresequenzen, die sich als Ergebnis des degenerierten genetischen Codes von der in SEQ ID NO: 1 ableiten
 - c) Derivate der in SEQ ID NO: 1 dargestellten Nukleinsäuresequenz, die für Polypeptide mit der in SEQ ID NO: 2
- 30 dargestellten Aminosäuresequenzen codieren und mindestens 50 % Homologie auf Aminosäureebene aufweisen, ohne daß die enzymatische Wirkung der Polypeptide wesentlich reduziert ist.
- 35 Erhöhung des Gehaltes von Fettsäuren, Ölen oder Lipiden mit $\Delta 6$ -Doppelbindungen bedeutet im Rahmen der vorliegenden Erfindung beispielsweise die künstlich erworbene Fähigkeit einer erhöhten Biosyntheseleistung durch funktionelle Überexpression des $\Delta 6$ -Desaturase-Gens in den erfindungsgemäßen Organismen vorteil-
- 40 haft in den erfindungsgemäßen transgenen Pflanzen gegenüber den nicht gentechnisch modifizierten Ausgangspflanzen zumindest für die Dauer mindestens einer Pflanzengeneration.

Der Biosyntheseort von Fettsäuren, Ölen oder Lipiden beispielsweise ist im allgemeinen der Samen oder Zellschichten des Samens, so daß eine samenspezifische Expression des $\Delta 6$ -Desaturase-Gens sinnvoll ist. Es ist jedoch naheliegend, daß die Biosynthese

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von Fettsäuren, Ölen oder Lipiden nicht auf das Samengewebe beschränkt sein muß, sondern auch in allen übrigen Teilen der Pflanze - beispielsweise in Epidermiszellen oder in den Knollen-gewebe spezifisch erfolgen kann.

5

Darüberhinaus ist eine konstitutive Expression des exogenen $\Delta 6$ -Desaturase-Gens von Vorteil. Andererseits kann aber auch eine induzierbare Expression wünschenswert erscheinen.

- 10 Die Wirksamkeit der Expression des $\Delta 6$ -Desaturase-Gens kann beispielsweise *in vitro* durch Sproßmeristemvermehrung ermittelt werden. Zudem kann eine in Art und Höhe veränderte Expression des $\Delta 6$ -Desaturase-Gens und deren Auswirkung auf die Fettsäure-, Öl- oder Lipidbiosyntheseleistung an Testpflanzen in Gewächshaus-
15 versuchen getestet werden.

Gegenstand der Erfindung sind wie oben beschrieben transgene Pflanzen, transformiert mit einer Nukleinsäuresequenz, die für eine $\Delta 6$ -Desaturase codiert, einem Vektor oder einer Expressions-

- 20 kassette enthaltend eine $\Delta 6$ -Desaturase-Gensequenz oder mit dieser hybridisierende DNA-Sequenzen, sowie transgene Zellen, Gewebe, Teile und Vermehrungsgut solcher Pflanzen. Besonders bevorzugt sind dabei transgene Kulturpflanzen wie oben beschrieben.

- 25 Pflanzen im Sinne der Erfindung sind mono- und dikotyle Pflanzen oder Algen.

Weitere Gegenstände der Erfindung sind:

- 30 - Verwendung einer $\Delta 6$ -Desaturase-DNA-Gensequenz mit der in SEQ ID NO:1 genannten Sequenz oder mit dieser hybridisierende DNA-Sequenzen zur Herstellung von Pilzen, Bakterien, Tieren oder Pflanzen bevorzugt Pflanzen mit erhöhtem Gehalt an Fettsäuren, Ölen oder Lipiden mit $\Delta 6$ -Doppelbindungen durch
35 Expression dieser $\Delta 6$ -Desaturase DNA-Sequenz in Pflanzen.
- Verwendung der Proteine mit den Sequenzen SEQ ID NO: 2 zur Herstellung von ungesättigten Fettsäuren in Pflanzen, Pilzen, Bakterien oder Tieren bevorzugt Pflanzen.

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Die Erfindung wird durch die folgenden Beispiele näher erläutert:

Beispiele

5 Beispiel 1: Allgemeine Klonierungsverfahren und Anzuchtungsverfahren:

Die Klonierungsverfahren wie z.B. Restriktionsspaltungen, Agarose-Gelelektrophorese, Reinigung von DNA-Fragmenten, Transfer
10 von Nukleinsäuren auf Nitrozellulose und Nylon Membranen, Verknüpfen von DNA-Fragmenten, Transformation von Escherichia coli Zellen, Anzucht von Organismen und die Sequenzanalyse rekombinanter DNA wurden wie bei Sambrook et al. (1989) (Cold Spring Harbor Laboratory Press: ISBN 0-87969-309-6) beschrieben durchgeführt.
15 Das Protonema von Physcomitrella patens (= P. patens) wurde in Flüssigmedium, wie von Reski et al. (Mol. Gen. Genet., 244, 1994: 352-359) beschrieben, angezogen.

Beispiel 2: Sequenzanalyse rekombinanter DNA

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Die Sequenzierung rekombinanter DNA-Moleküle erfolgte mit einem Laserfluoreszenz-DNA-Sequenzierer der Firma ABI nach der Methode von Sanger (Sanger et al. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467). Fragmente resultierend aus einer Polymerase Ketten-
25 reaktion wurden zur Vermeidung von Polymerasefehlern in zu exprimierenden Konstrukten sequenziert und überprüft.

Beispiel 3: Lipidanalyse aus dem Protonema von P. patens und aus Hefezellen

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Die Lipide wurden mit Chloroform/Methanol wie bei Siebertz et al. (Eur. J. Biochem., 101, 1979: 429-438) beschrieben aus dem Protonema von S. patens oder aus Hefezellen extrahiert und über Dünnschichtchromatographie (= TLC) mit Diethylether ge-
35 reinigt. Die erhaltenen Fettsäuren wurden zu den entsprechenden Methylestern transmethyliert und mit Gaschromatographie (= GC) analysiert. Die verschiedenen Methylester wurden mit den entsprechenden Standards identifiziert. Entsprechende Fettsäurepyrrolididen wurden, wie bei Anderson et al. (Lipids, 9, 1974:
40 185-190) beschrieben, erhalten und mit GC-MS bestimmt.

Beispiel 4: Funktionelle Expression der $\Delta 6$ -Desaturase cDNA von *P. patens* in Hefen

Die Expression-Experimente in Hefen wurden mit PPDES6-cDNA durchgeführt. Knock-out-Experimente hatten gezeigt (Daten und Versuchsdurchführung nicht gezeigt bzw. beschrieben), daß der Knock-out zu einem Verlust an 20:3^{11,14,17}-, 20:4^{5,8,11,14}-, 20:4^{5,11,14,17}- und 20:5^{5,8,11,14,17}-Fettsäuren führt. Gleichzeitig steigen die 18:2^{9,12}- und 18:3^{9,12,15}-Fettsäuren an. Für die Expression in Hefe wurde der PPDES6-cDNA in den Hefe-Expressionsvektor pYES2 (Invitrogen) subkloniert. Der erhaltene Vektor erhielt die Bezeichnung pYESdelta6. Mit pYES2 (Kontrolle) und pYESdelta6 ($\Delta 6$ -Desaturase-cDNA) transformierte Hefekulturen wurden auf Uracil-dop-out Medium mit 2 % Raffinose und 1 % Tergitol NP-40 (zur Stabilisierung der Fettsäuren) angezogen. Für die Expression wurden die Zellen mit Galactose (Endkonzentration 2 %) bis zu einer optischen Dichte (= OD) von 0,5 bei 600nm angezogen. In Fütterungsexperimenten wurden Fettsäuren in 5 % Tergitol solubilisiert und mit einer Endkonzentration von 0,0003 % zugesetzt. Die Ergebnisse der Expression sind Tabelle I zu entnehmen. Die Synthese von Fettsäuren mit einer Doppelbindung an Position 6 ist nur in Gegenwart des Expressionskonstrukts mit der $\Delta 6$ -Desaturase-cDNA möglich. Dieses $\Delta 6$ -Desaturase-Enzym hat eine größere Aktivität gegenüber Fettsäuren, die schon eine Doppelbindung an Position 9 oder 12 (Bezug auf Kohlenstoffatom in der Kette) enthalten. Es wurden die Fettsäuremethylester des gesamten Lipids der Hefen mit GC analysiert. Die einzelnen synthetisierten Fettsäuren werden in der Tabelle in Mol-% der gesamten Fettsäuren angegeben.

30 Tabelle I: Fettsäurezusammensetzung in transformierten Hefen gegenüber der Kontrolle

Gesamt Fettsäure (%)				
	pYES2		pYESdelta6	
35 Fettsäuren	-	-	+ 18:2 ^{9,12}	+18:3 ^{9,12,15}
16:0	16,4	16,1	23,8	25,8
16:1 ⁹	54,0	55,5	38,1	31,4
16:2 ^{6,9}	-	4,2	1,7	-
40 18:0	3,2	2,4	4,0	-
18:1 ⁹	24,9	19,7	19,1	19,2
18:2 ^{6,9}	-	0,6	0,2	-
18:2 ^{9,12}	-	-	8,5	-
45 18:3 ^{6,9,12}	-	-	4,0	-
18:3 ^{9,12,15}	-	-	-	11,7
18:4 ^{6,9,12,15}	-	-	-	3,0

Beispiel 5: Transformation von *P. patens*

- Die Polyethylenglycol vermittelte direkte DNA-Transformation von Protoplasten wurde, wie von Schäfer et al. (Mol. Gen. Genet., 5 226, 1991: 418-424) beschrieben, durchgeführt. Die Selektion der Transformanten erfolgte auf G418-enthaltenden Medium (Girke et al., The Plant Journal, 15, 1998: 39-48).

10 Beispiel 6: Isolierung von $\Delta 6$ -Desaturase cDNA und genomischen Clonen von *P. patens*

Mit Hilfe eines PCR-Ansatzes mit den folgenden degenerierten Oligonukleotiden als Primer:

- 15 A: TGGTGGAA(A/G)TGGA(C/A)ICA(T/C)AA und
B: GG(A/G)AA(A/C/G/T)A(A/G)(G/A)TG(G/A)TG(C/T)TC]

und dem folgenden Temperaturprogramm:

- 94°C, 3 min; [94°C, 20 sec; 45°C, 30 sec; 72°C, 1 min], 30 Zyklen;
20 72°C, 5 min, wurden schließlich Fragmente einer $\Delta 6$ -Desaturase-Gen kloniert. Für die Klonierung wurde poly(A)RNA aus 12 Tage alten *P. patens* Protonema-Kultur isoliert. Mit dieser poly(A)RNA wurde die oben beschriebene PCR durchgeführt. Fragmente der erwarteten Fragmentlänge (500 bis 600 bp) wurden in pUC18 kloniert und
25 sequenziert. Die abgeleitete Aminosäuresequenz eines PCR-Fragments zeigte Ähnlichkeiten zu bekannten $\Delta 6$ -Desaturasen. Da bekannt war, daß *P. patens* eine $\Delta 6$ -Desaturase besitzt, wurde angenommen, daß dieser Klon für einen Teil einer $\Delta 6$ -Desaturase codiert.
- 30 Ein vollständiger cDNA-Klon (= PPDES6-cDNA) wurde aus einer *P. patens* cDNA-Bank von 12 Tage alten Protonemata mit Hilfe des oben genannten PCR-Fragments isoliert. Die Nukleotidsequenz wird in SEQ ID NO:1 wiedergegeben. Die abgeleitete Aminosäuresequenz ist SEQ ID NO:2 zu entnehmen. Die zugehörige genomische Sequenz
35 (= PPDES6-Gen) konnte mit Hilfe der PCR und den folgenden Oligonukleotiden als Primer isoliert werden:

C: CCGAGTCGCGGATCAGCC

D: CAGTACATTCGGTCATTCACC:

40

- Tabelle II gibt die Ergebnisse des Vergleichs zwischen der neuen *P. patens* $\Delta 6$ -Desaturase über die gesamte Nukleinsäuresequenz mit folgenden bekannten $\Delta 6$ -Desaturase wieder: *Borago officinalis* (U79010), *Synechocystis* sp (L11421), *Spirulina platensis* (X87094), *Caenorhabditis elegans* (AF031477), *Mortierella alpina* (WO 98/46764), *Homo sapiens* (Cho et al., J. Biol. Chem., 274, 1999: 471-477), *Rattus norvegicus* (AB021980) und *Mus musculus*

(Cho et al., J. Biol. Chem., 274, 1999: 471-477). Die Analyse wurde mit dem Gap Programm (GCG-Package, Version 9,1) und den folgenden Analysenparametern durchgeführt: scoring matrix, blosum62, gap creation penalty, 12; gap extension penalty, 4.

- 5 Die Ergebnisse geben die bestimmte Identität oder Ähnlichkeit [] in Prozent (%) im Vergleich zur *P. patens*-Sequenz wieder.

Tabelle II: Sequenzvergleich zwischen *P. patens* $\Delta 6$ -Desaturase und anderen $\Delta 6$ -Desaturasen

10

Sequenz	Aminosäuresequenz-Identität [Ähnlichkeit] (%)
Borago officinalis	31 [38]
Synechocystis sp.	21 [29]
15 Spirulina platensis	20 [29]
Caenorhabditis elegans	35 [43]
Mortierella alpina	39 [47]
Homo sapiens	27 [38]
20 Rattus norvegicus	28 [39]
Mus musculus	29 [39]

Beispiel 7: Klonierung der $\Delta 6$ -Desaturase aus *Physcomitrella*
 25 *patens*

Die genomische $\Delta 6$ -Acyllipid-Desaturase aus *Physcomitrella patens* wurde auf Grundlage der veröffentlichten Sequenz (Girke et al., Plant J., 15, 1998: 39-48) mittels Polymerasekettenreaktion und
 30 Klonierung modifiziert, isoliert und für das erfindungsgemäße Verfahren eingesetzt. Dazu wurde zunächst mittels Polymerasekettenreaktion unter Verwendung von zwei genspezifischen Primern ein Desaturase-Fragment isoliert und in das bei Girke et al. (siehe oben) beschriebene Desaturasegen eingesetzt.

35

Primer TG5: 5'-ccgctcgagcgaggttgttggtggagcggc und
 Primer TG3: 5'-ctgaaatagtcttgctcc-3'

dienten zunächst zur Amplifizierung eines Genfragmentes mittels
 40 Polymerasekettenreaktion (30 Zyklen, 30 sek. 94° V, 30 sek. 50°C, 60 sek. 72°C, 10 min Nachinkubation bei 72°C, in einem Perkin Elmer Thermocycler).

45

- a) Klonierung eines Expressionsplasmids, das die $\Delta 6$ -Desaturase unter Kontrolle des 35S CaMV Promotors exprimiert:

5 Durch Primer TG5 wurde eine XhoI Schnittstelle in das Fragment eingeführt. Ein XhoI/Eco47III Fragment wurde durch Restriktion erhalten und in die bei Girke et al. beschriebene PPDES6-Gensequenz nach analoger Restriktion mit XhoI/Eco47III ausgetauscht. Das Konstrukt erhielt den Namen pZK. Das Insert von pZK wurde als XhoI/HindIII Fragment nach Auffüllen der
10 HindIII-Schnittstelle mit Nukleotiden durch Behandlung mit dem Klenow Fragment der DNA Polymerase I in die XhoI/SmaI Schnittstellen von pRT99/35S kloniert. Das resultierende Plasmid pSK enthält den 35S-Promotor [Cauliflower-Mosaik-Virus, Franck et al. (1980) Cell 21, 285], die $\Delta 6$ -Desaturase
15 aus Moos und den 35S-Terminator im Vektor pRT.

- b) Konstruktion eines Expressionskonstruktes unter Kontrolle des Napin-Promotors:

20 Durch Schneiden des Plasmides pSK mit XhoI, Behandlung mit T4-DNA Polymerase und PstI-Restriktion wurde das erhaltene Promotor-Desaturase-Fragment mit Terminator in den Vektor pJH3 kloniert. Dazu wurde der Vektor BamHI geschnitten und mit Klenow-Enzym die Überhänge aufgefüllt sowie anschließend
25 mit PstI nachgeschnitten. Es entstand durch Ligation des Desaturase-Terminator-Fragmentes in den Vektor das Plasmid pJH7, das einen Napin-Promotor beinhaltet (Scofield et al., 1987, J. Biol. Chem. 262, 12202-8). Die Expressionskassette aus pJH7 wurde mit Bsp120I und NotI geschnitten und in den
30 binären Vektor pRE kloniert. Es entstand das Plasmid pRE-Ppdes6.

In einer PCR Reaktion wurde die erfindungsgemäße $\Delta 6$ -Desaturase cDNA aus *P. patens* als Matrize verwendet.
35 Mithilfe der nachfolgend aufgeführten Oligonukleotide wurde eine BamHI-Restriktionsschnittstelle vor dem Startcodon und drei Adeninnukleotide als Konsensustranslationssequenz für Eukaryoten in die $\Delta 6$ -Desaturase cDNA eingeführt. Es wurde
40 ein 1512 Basenpaarfragment der $\Delta 6$ -Desaturase amplifiziert und sequenziert.

Pp-d6Des1: 5'- CC GGTACC aaaatggtattcgcgggcggtg -3'

Pp-d6Des2: 3'- CC GGTACC ttaactggtggttagcatgct -3'

45 Die Reaktionsgemische enthielten ca. 1 ng/micro l Matrizen DNA, 0,5 μ M der Oligonukleotide und, 200 μ M Desoxy-Nukleotide (Pharmacia), 50 mM KCl, 10 mM Tris-HCl (pH 8,3 bei 25°C,

1,5 mM $MgCl_2$) und 0,02 U/ μ l Pwo Polymerase (Boehringer Mannheim) und werden in einer PCR-Maschine der Firma Perkin Elmer mit folgendem Temperaturprogramm inkubiert:

- | | | |
|---|---------------------------|--------------|
| 5 | Anlagerungstemperatur: | 50°C, 30 sec |
| | Denaturierungstemperatur: | 95°C, 30 sec |
| | Elongationstemperatur: | 72°C, 90 sec |
| | Anzahl der Zyklen: | 30 |

- 10 c) Konstruktion eines Expressionskonstruktes unter Kontrolle des USP-Promotors:

Das erhaltene Fragment von ca. 1,5 kB Basenpaaren wurde in den mit EcoRV gespaltenen Vektor pBluescript SK- (Stratagene) ligiert und stand für weitere Klonierungen als BamHI Fragment zur Verfügung.

- 15
- Für die Transformation von Pflanzen wurde ein weiterer Transformationsvektor auf Basis von pBin-USP erzeugt, der das BamHI-Fragment der $\Delta 6$ -Desaturase enthält. pBin-USP ist ein
- 20 Derivat des Plasmides pBin19. pBinUSP entstand aus pBin19, indem in pBin19 [Bevan et al. (1980) Nucl. Acids Res. 12, 8711] ein USP-Promotor als EcoRI-BamHI-Fragment inseriert wurde. Das Polyadenylierungssignal ist das des Gens 3 der
- 25 T-DNA des Ti-Plasmides pTiACH5 (Gielen et al., (1984) EMBO J. 3, 835), wobei Nukleotide 11749-11939 als PvuII-HindIII-Fragment isoliert und nach Addition von SphI-Linkern an die PvuII-Schnittstelle zwischen die SphI-HindIII Schnittstelle des Vektors kloniert. Der USP-Promotor entspricht den Nukleotiden 1-684 (Genbank Accession X56240), wobei ein Teil der
- 30 nichtcodierenden Region des USP-Gens im Promotor enthalten ist. Das 684 Basenpaar große Promotorfragment wurde mittels käuflichen T7-Standardprimer (Stratagene) und mit Hilfe eines synthetisierten Primers über eine PCR-Reaktion nach Standardmethoden amplifiziert (Primersequenz: 5'-GTCGACCCGCGGACTAGTG-
- 35 GGCCCTCTAGACCCGGGGGATCC GGATCTGCTGGCTATGAA-3'). Das PCR-Fragment wurde mit EcoRI/SalI nachgeschnitten und in den Vektor pBin19 mit OCS Terminator eingesetzt. Es entstand das Plasmid mit der Bezeichnung pBinUSP.

- 40 d) Konstruktion eines Expressionskonstruktes unter Kontrolle des vATPase-C1-Promotors aus Beta vulgaris:

- Analog zum Expressionsplasmid mit dem USP-Promotor wurde ein Konstrukt unter Verwendung des v-ATPase-c1-Promotors
- 45 erstellt. Der Promotor wurde als EcoRI/KpnI Fragment in das Plasmid pBin19 mit OCS Terminator kloniert und über BamHI das $\Delta 6$ -Desaturasegen aus *P. patens* zwischen Promotor und

Terminator inseriert. Der Promotor entspricht einem 1153 Basenpaarfragment aus beta-Vulgaris (Plant Mol Biol, 1999, 39:463-475).

- 5 Das Konstrukt wurde zur Transformation von Arabidopsis thaliana und Rapspflanzen eingesetzt.

Beispiel 8: Erzeugung transgener Rapspflanzen (verändert nach Moloney et al., 1992, Plant Cell Reports, 8:238-242)

10

- Zur Erzeugung transgener Rapspflanzen wurden binäre Vektoren in Agrobacterium tumefaciens C58C1:pgV2260 oder Escherichia coli genutzt (Deblaere et al, 1984, Nucl. Acids. Res. 13, 4777-4788). Zur Transformation von Rapspflanzen (Var. Drakkar, NPZ Nord-
15 deutsche Pflanzenzucht, Hohenlieth, Deutschland), wurde eine 1:50 Verdünnung einer Übernachtskultur einer positiv transformierten Agrobakterienkolonie in Murashige-Skoog Medium (Murashige und
20 Skoog 1962 Physiol. Plant. 15, 473) mit 3 % Saccharose (3MS-Medium) benutzt. Petiolen oder Hypokotyledonen frisch gekeimter steriler Rapspflanzen (zu je ca. 1 cm²) wurden in einer Petri-
schale mit einer 1:50 Agrobakterienverdünnung für 5-10 Minuten inkubiert. Es folgte eine 3-tägige Inkubation in Dunkelheit bei 25°C auf 3MS-Medium mit 0,8 % Bacto-Agar. Die Kultivierung wurde
25 nach 3 Tagen mit 16 Stunden Licht/8 Stunden Dunkelheit weitergeführt und in wöchentlichem Rhythmus auf MS-Medium mit 500 mg/l Claforan (Cefotaxime-Natrium), 50 mg/l Kanamycin, 20 µM Benzyl-
aminopurin (BAP) und 1,6 g/l Glukose weitergeführt. Wachsende Sprosse wurden auf MS-Medium mit 2 % Saccharose, 250 mg/l Claforan und 0,8 % Bacto-Agar überführt. Bildeten sich nach
30 drei Wochen keine Wurzeln, so wurde als Wachstumshormon 2-Indolbuttersäure zum Bewurzeln zum Medium zugegeben.

- Regenerierte Sprosse wurden auf 2MS-Medium mit Kanamycin und Claforan erhalten, nach Bewurzelung in Erde überführt und
35 nach Kultivierung für zwei Wochen in einer Klimakammer oder im Gewächshaus angezogen, zur Blüte gebracht, reife Samen geerntet und auf Δ6-Desaturase-Expression mittels Lipidanalysen untersucht. Linien mit erhöhten Gehalten an oder Doppelbindungen an der Δ6-Position wurden identifiziert. Es konnte in den stabil
40 transformierten transgenen Linien, die das Transgen funktionell exprimierten, ein erhöhter Gehalt von Doppelbindungen an der Δ6-Position im Vergleich zu untransformierten Kontrollpflanzen festgestellt werden.

45

Beispiel 8: Lipidextraktion aus Samen

Das Pflanzenmaterial wurde zunächst mechanisch durch Mörsern homogenisiert, um es einer Extraktion zugänglicher zu machen.

5

Dann wurde es 10 min bei 100°C abgekocht und nach dem Abkühlen auf Eis sedimentiert. Das Zellsediment wurde mit 1 N methanolischer Schwefelsäure und 2 % Dimethoxypropan 1h bei 90°C hydrolysiert und die Lipide transmethyliert. Die resultierenden Fettsäure-

- 10 methylester (FAME) wurden in Petrolether extrahiert. Die extrahierten FAME wurden durch Gasflüssigkeitschromatographie mit einer Kapillarsäule (Chrompack, WCOT Fused Silica, CP-Wax-52 CB, 25 m, 0,32 mm) und einem Temperaturgradienten von 170°C auf 240°C in 20 min und 5 min bei 240°C analysiert. Die Identität der Fettsäuremethylester wurde durch Vergleich mit entsprechenden FAME-Standards (Sigma) bestätigt. Die Identität und die Position der Doppelbindung konnte durch geeignete chemische Derivatisierung der FAME-Gemische z.B. zu 4,4-Dimethoxyoxazolin-Derivaten (Christie, 1997, in: Advances in Lipid Methodology, 4. Auflage: Christie, Oily Press, Dundee, 119-169, und 1998, Gaschromatographie-Massenspektrometrie Verfahren, Lipide 33:343-353) mittels GC-MS weiter analysiert werden. Die GC-Analysen der Fettsäuremethylester aus den transgenen Rapssamen, die samenspezifisch die $\Delta 6$ -Desaturase exprimierten sind in Tabelle III dargestellt. Die 25 transgenen Rapssamen weisen mindestens 4,95 % γ -Linolensäure im Samen auf.

Tabelle III gibt die GC-Analysen der Fettsäuremethylester aus reifen, transgenen Rapssamen, die $\Delta 6$ -Desaturase samen-

- 30 spezifisch exprimieren, wieder. Die Fettsäurezusammensetzung ist in [mol %] der Gesamtfettsäuren angegeben. Es ist festzustellen, daß einzelne Pflanzen der T2 Generation, die aus positiv transformierten und geselbsteten Pflanzen erhalten wurden, bis zu ca. 4,95 % γ -Linolensäure enthalten.

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Tabelle III: GC-Analysen der Fettsäuremethylester von Raps

	Bezeichnung	18:0	18:1	18:2	18:3 (γ)	18:3 (α)	18:4
5	R2-T2-11/1a	1,98	53,58	22,63	3,86	11,38	0
	R2-T2-11/1b	1,86	52,04	25,45	2,31	11,39	0
	R2-T2-11/1c	1,95	49,17	24,30	2,84	9,20	0
	R2-T2-11/3	1,82	49,83	24,54	3,88	10,12	0
	R2-T2-11/4	1,72	48,02	24,66	4,95	9,52	0
	R2-T2-11/5a	1,73	51,98	25,27	4,27	9,61	0
10	R2-T2-11/5b	2,02	56,19	25,08	0	9,33	0
	R2-T2-11/5c	2,01	46,95	27,38	0	10,37	0
	R2-T2-11/5d	1,83	49,49	24,15	4,40	8,65	0
	R2-T2-11/6	2,08	54,52	23,94	2,05	9,29	0
	R2-T2-11/10	1,94	53,92	22,81	4,06	9,44	0
15	R2-T2-WT	1,90	47,75	30,91	0	10,51	0

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Patentansprüche

1. Verfahren zur Herstellung von ungesättigten Fettsäuren, da-
5 durch gekennzeichnet, daß mindestens eine isolierte Nuklein-
säuresequenz, die für ein Polypeptid mit $\Delta 6$ -Desaturase-
aktivität codiert, ausgewählt aus der Gruppe:
 - a) einer Nukleinsäuresequenz mit der in SEQ ID NO: 1 dar-
10 gestellten Sequenz,
 - b) Nukleinsäuresequenzen, die sich als Ergebnis des
degenerierten genetischen Codes von der in SEQ ID NO: 1
15 ableiten
 - c) Derivate der in SEQ ID NO: 1 dargestellten Nukleinsäure-
sequenz, die für Polypeptide mit der in SEQ ID NO: 2 dar-
20 gestellten Aminosäuresequenzen codieren und mindestens
50 % Homologie auf Aminosäureebene aufweisen, ohne daß
die enzymatische Wirkung der Polypeptide wesentlich
reduziert ist,

in einen Organismus eingebracht wird, dieser Organismus
angezogen wird, wobei der angezogene Organismus mindestens
25 1 Mol-% ungesättigte Fettsäuren bezogen auf den gesamten
Fettsäuregehalt im Organismus enthält.
2. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß die
Nukleinsäuresequenz von einer Pflanze oder Alge stammt.
30
3. Verfahren nach Anspruch 1 oder 2, dadurch gekennzeichnet,
daß die Nukleinsäuresequenz von *Physcomitrella patens* stammt.
4. Verfahren nach den Ansprüchen 1 bis 3, dadurch gekenn-
35 zeichnet, daß es sich bei dem Organismus um ein organismus
ausgewählt aus der Gruppe Bakterium, Pilz, Ciliat, Alge,
Cyanobakterium, Tier oder Pflanze handelt.
5. Verfahren nach den Ansprüchen 1 bis 4, dadurch gekenn-
40 zeichnet, daß es sich bei dem Organismus um eine Pflanze
oder Alge handelt.

6. Verfahren nach den Ansprüchen 1 bis 5, dadurch gekennzeichnet, daß es sich bei dem Organismus um eine Ölfruchtpflanzen handelt.
- 5 7. Verfahren nach den Ansprüchen 1 bis 6, dadurch gekennzeichnet, daß der angezogene Organismus mindestens 5 Gew-% ungesättigte Fettsäuren bezogen auf den gesamten Fettsäuregehalt im Organismus enthält.
- 10 8. Verfahren nach den Ansprüchen 1 bis 7, dadurch gekennzeichnet, daß die ungesättigten Fettsäuren aus dem Organismus isoliert werden.
9. Transgener Organismus ausgewählt aus der Gruppe Pflanzen,
15 Pilze, Ciliaten, Algen, Bakterien, Cyanobakterien oder Tiere, die mindestens eine isolierte Nukleinsäuresequenz enthalten, die für ein Polypeptid mit $\Delta 6$ -Desaturaseaktivität codiert, ausgewählt aus der Gruppe:
- 20 a) einer Nukleinsäuresequenz mit der in SEQ ID NO: 1 dargestellten Sequenz,
- b) Nukleinsäuresequenzen, die sich als Ergebnis des degenerierten genetischen Codes von der in SEQ ID NO: 1
25 ableiten
- c) Derivate der in SEQ ID NO: 1 dargestellten Nukleinsäuresequenz, die für Polypeptide mit der in SEQ ID NO: 2 dargestellten Aminosäuresequenzen codieren und mindestens
30 50 % Homologie auf Aminosäureebene aufweisen, ohne daß die enzymatische Wirkung der Polypeptide wesentlich reduziert ist.
10. Transgener Organismus nach Anspruch 9, dadurch gekennzeichnet,
35 zeichnet, daß es sich bei dem Organismus um eine Pflanze oder Alge handelt.
11. Öl, Lipide oder Fettsäuren oder eine Fraktion davon, hergestellt durch das Verfahren nach einem der Ansprüche 1
40 bis 8.
12. Verwendung der Öl-, Lipid- oder Fettsäurezusammensetzung nach Anspruch 11 oder transgene Organismen nach Anspruch 9 in Futtermitteln, Nahrungsmitteln, Kosmetika oder Pharmazeutika.
45

SEQUENZPROTOKOLL

<170> PatentIn Vers. 2.0

<210> 1

<211> 2012

<212> DNA

<213> Physcomitrella patens

<220>

<221> CDS

<222> (319)..(1896)

<400> 1

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tgtggagcgg cttttgaa atg gta ttc gcg ggc ggt gga ctt cag cag ggc 351
          Met Val Phe Ala Gly Gly Gly Leu Gln Gln Gly
          .1              5              10

tct ctc gaa gaa aac atc gac gtc gag cac att gcc agt atg tct ctc 399
Ser Leu Glu Glu Asn Ile Asp Val Glu His Ile Ala Ser Met Ser Leu
          15              20              25

ttc agc gac ttc ttc agt tat gtg tct tca act gtt ggt tcg tgg agc 447
Phe Ser Asp Phe Phe Ser Tyr Val Ser Ser Thr Val Gly Ser Trp Ser
          30              35              40

gta cac agt ata caa cct ttg aag cgc ctg acg agt aag aag cgt gtt 495
Val His Ser Ile Gln Pro Leu Lys Arg Leu Thr Ser Lys Lys Arg Val
          45              50              55

tcg gaa agc gct gcc gtg caa tgt ata tca gct gaa gtt cag aga aat 543
Ser Glu Ser Ala Ala Val Gln Cys Ile Ser Ala Glu Val Gln Arg Asn
          60              65              70              75

tcg agt acc cag gga act gcg gag gca ctc gca gaa tca gtc gtg aag 591
Ser Ser Thr Gln Gly Thr Ala Glu Ala Leu Ala Glu Ser Val Val Lys
          80              85              90

ccc acg aga cga agg tca tct cag tgg aag aag tcg aca cac ccc cta 639
Pro Thr Arg Arg Arg Ser Ser Gln Trp Lys Lys Ser Thr His Pro Leu
          95              100              105

```



tca gaa gta gca gta cac aac aag cca agc gat tgc tgg att gtt gta	687
Ser Glu Val Ala Val His Asn Lys Pro Ser Asp Cys Trp Ile Val Val	
110 115 120	
aaa aac aag gtg tat gat gtt tcc aat ttt gcg gac gag cat ccc gga	735
Lys Asn Lys Val Tyr Asp Val Ser Asn Phe Ala Asp Glu His Pro Gly	
125 130 135	
gga tca gtt att agt act tat ttt gga cga gac ggc aca gat gtt ttc	783
Gly Ser Val Ile Ser Thr Tyr Phe Gly Arg Asp Gly Thr Asp Val Phe	
140 145 150 155	
tct agt ttt cat gca gct tct aca tgg aaa att ctt caa gac ttt tac	831
Ser Ser Phe His Ala Ala Ser Thr Trp Lys Ile Leu Gln Asp Phe Tyr	
160 165 170	
att ggt gac gtg gag agg gtg gag ccg act cca gag ctg ctg aaa gat	879
Ile Gly Asp Val Glu Arg Val Glu Pro Thr Pro Glu Leu Leu Lys Asp	
175 180 185	
ttc cga gaa atg aga gct ctt ttc ctg agg gag caa ctt ttc aaa agt	927
Phe Arg Glu Met Arg Ala Leu Phe Leu Arg Glu Gln Leu Phe Lys Ser	
190 195 200	
tcg aaa ttg tac tat gtt atg aag ctg ctc acg aat gtt gct att ttt	975
Ser Lys Leu Tyr Tyr Val Met Lys Leu Leu Thr Asn Val Ala Ile Phe	
205 210 215	
gct gcg agc att gca ata ata tgt tgg agc aag act att tca gcg gtt	1023
Ala Ala Ser Ile Ala Ile Ile Cys Trp Ser Lys Thr Ile Ser Ala Val	
220 225 230 235	
ttg gct tca gct tgt atg atg gct ctg tgt ttc caa cag tgc gga tgg	1071
Leu Ala Ser Ala Cys Met Met Ala Leu Cys Phe Gln Gln Cys Gly Trp	
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cta tcc cat gat ttt ctc cac aat cag gtg ttt gag aca cgc tgg ctt	1119
Leu Ser His Asp Phe Leu His Asn Gln Val Phe Glu Thr Arg Trp Leu	
255 260 265	
aat gaa gtt gtc ggg tat gtg atc ggc aac gcc gtt ctg ggg ttt agt	1167
Asn Glu Val Val Gly Tyr Val Ile Gly Asn Ala Val Leu Gly Phe Ser	
270 275 280	
aca ggg tgg tgg aag gag aag cat aac ctt cat cat gct gct cca aat	1215
Thr Gly Trp Trp Lys Glu Lys His Asn Leu His His Ala Ala Pro Asn	
285 290 295	
gaa tgc gat cag act tac caa cca att gat gaa gat att gat act ctc	1263
Glu Cys Asp Gln Thr Tyr Gln Pro Ile Asp Glu Asp Ile Asp Thr Leu	



300	305	310	315	
ccc ctc att gcc tgg agc aag gac ata ctg gcc aca gtt gag aat aag				1311
Pro Leu Ile Ala Trp Ser Lys Asp Ile Leu Ala Thr Val Glu Asn Lys				
320		325	330	
aca ttc ttg cga atc ctc caa tac cag cat ctg ttc ttc atg ggt ctg				1359
Thr Phe Leu Arg Ile Leu Gln Tyr Gln His Leu Phe Phe Met Gly Leu				
335		340	345	
tta ttt ttc gcc cgt ggt agt tgg ctc ttt tgg agc tgg aga tat acc				1407
Leu Phe Phe Ala Arg Gly Ser Trp Leu Phe Trp Ser Trp Arg Tyr Thr				
350		355	360	
tct aca gca gtg ctc tca cct gtc gac agg ttg ttg gag aag gga act				1455
Ser Thr Ala Val Leu Ser Pro Val Asp Arg Leu Leu Glu Lys Gly Thr				
365		370	375	
gtt ctg ttt cac tac ttt tgg ttc gtc ggg aca gcg tgc tat ctt ctc				1503
Val Leu Phe His Tyr Phe Trp Phe Val Gly Thr Ala Cys Tyr Leu Leu				
380		385	390	395
cct ggt tgg aag cca tta gta tgg atg gcg gtg act gag ctc atg tcc				1551
Pro Gly Trp Lys Pro Leu Val Trp Met Ala Val Thr Glu Leu Met Ser				
400		405	410	
ggc atg ctg ctg ggc ttt gta ttt gta ctt agc cac aat ggg atg gag				1599
Gly Met Leu Leu Gly Phe Val Phe Val Leu Ser His Asn Gly Met Glu				
415		420	425	
gtt tat aat tcg tct aaa gaa ttc gtg agt gca cag atc gta tcc aca				1647
Val Tyr Asn Ser Ser Lys Glu Phe Val Ser Ala Gln Ile Val Ser Thr				
430		435	440	
cgg gat atc aaa gga aac ata ttc aac gac tgg ttc act ggt ggc ctt				1695
Arg Asp Ile Lys Gly Asn Ile Phe Asn Asp Trp Phe Thr Gly Gly Leu				
445		450	455	
aac agg caa ata gag cat cat ctt ttc cca aca atg ccc agg cat aat				1743
Asn Arg Gln Ile Glu His His Leu Phe Pro Thr Met Pro Arg His Asn				
460		465	470	475
tta aac aaa ata gca cct aga gtg gag gtg ttc tgt aag aaa cac ggt				1791
Leu Asn Lys Ile Ala Pro Arg Val Glu Val Phe Cys Lys Lys His Gly				
480		485	490	
ctg gtg tac gaa gac gta tct att gct acc ggc act tgc aag gtt ttg				1839
Leu Val Tyr Glu Asp Val Ser Ile Ala Thr Gly Thr Cys Lys Val Leu				
495		500	505	
aaa gca ttg aag gaa gtc gcg gag gct gcg gca gag cag cat gct acc				1887



4

Lys Ala Leu Lys Glu Val Ala Glu Ala Ala Glu Gln His Ala Thr
 510 515 520

acc agt taa cagtcctttgg aaagcttggc aattgatctt tattctccac 1936
 Thr Ser
 525

ggcagttgct tgtttgtttt ggggtgaatg accgaatgta ctggcatcca ttcttctgta 1996
 gccatcaatt ttgaac 2012

<210> 2

<211> 525

<212> PRT

<213> Physcomitrella patens

<400> 2

Met Val Phe Ala Gly Gly Gly Leu Gln Gln Gly Ser Leu Glu Glu Asn
 1 5 10 15

Ile Asp Val Glu His Ile Ala Ser Met Ser Leu Phe Ser Asp Phe Phe
 20 25 30

Ser Tyr Val Ser Ser Thr Val Gly Ser Trp Ser Val His Ser Ile Gln
 35 40 45

Pro Leu Lys Arg Leu Thr Ser Lys Lys Arg Val Ser Glu Ser Ala Ala
 50 55 60

Val Gln Cys Ile Ser Ala Glu Val Gln Arg Asn Ser Ser Thr Gln Gly
 65 70 75 80

Thr Ala Glu Ala Leu Ala Glu Ser Val Val Lys Pro Thr Arg Arg Arg
 85 90 95

Ser Ser Gln Trp Lys Lys Ser Thr His Pro Leu Ser Glu Val Ala Val
 100 105 110

His Asn Lys Pro Ser Asp Cys Trp Ile Val Val Lys Asn Lys Val Tyr
 115 120 125

Asp Val Ser Asn Phe Ala Asp Glu His Pro Gly Gly Ser Val Ile Ser
 130 135 140

Thr Tyr Phe Gly Arg Asp Gly Thr Asp Val Phe Ser Ser Phe His Ala
 145 150 155 160

Ala Ser Thr Trp Lys Ile Leu Gln Asp Phe Tyr Ile Gly Asp Val Glu
 165 170 175



5

Arg Val Glu Pro Thr Pro Glu Leu Leu Lys Asp Phe Arg Glu Met Arg
 180 185 190

Ala Leu Phe Leu Arg Glu Gln Leu Phe Lys Ser Ser Lys Leu Tyr Tyr
 195 200 205

Val Met Lys Leu Leu Thr Asn Val Ala Ile Phe Ala Ala Ser Ile Ala
 210 215 220

Ile Ile Cys Trp Ser Lys Thr Ile Ser Ala Val Leu Ala Ser Ala Cys
 225 230 235 240

Met Met Ala Leu Cys Phe Gln Gln Cys Gly Trp Leu Ser His Asp Phe
 245 250 255

Leu His Asn Gln Val Phe Glu Thr Arg Trp Leu Asn Glu Val Val Gly
 260 265 270

Tyr Val Ile Gly Asn Ala Val Leu Gly Phe Ser Thr Gly Trp Trp Lys
 275 280 285

Glu Lys His Asn Leu His His Ala Ala Pro Asn Glu Cys Asp Gln Thr
 290 295 300

Tyr Gln Pro Ile Asp Glu Asp Ile Asp Thr Leu Pro Leu Ile Ala Trp
 305 310 315 320

Ser Lys Asp Ile Leu Ala Thr Val Glu Asn Lys Thr Phe Leu Arg Ile
 325 330 335

Leu Gln Tyr Gln His Leu Phe Phe Met Gly Leu Leu Phe Phe Ala Arg
 340 345 350

Gly Ser Trp Leu Phe Trp Ser Trp Arg Tyr Thr Ser Thr Ala Val Leu
 355 360 365

Ser Pro Val Asp Arg Leu Leu Glu Lys Gly Thr Val Leu Phe His Tyr
 370 375 380

Phe Trp Phe Val Gly Thr Ala Cys Tyr Leu Leu Pro Gly Trp Lys Pro
 385 390 395 400

Leu Val Trp Met Ala Val Thr Glu Leu Met Ser Gly Met Leu Leu Gly
 405 410 415

Phe Val Phe Val Leu Ser His Asn Gly Met Glu Val Tyr Asn Ser Ser
 420 425 430

Lys Glu Phe Val Ser Ala Gln Ile Val Ser Thr Arg Asp Ile Lys Gly
 435 440 445



6

Asn Ile Phe Asn Asp Trp Phe Thr Gly Gly Leu Asn Arg Gln Ile Glu
450 455 460

His His Leu Phe Pro Thr Met Pro Arg His Asn Leu Asn Lys Ile Ala
465 470 475 480

Pro Arg Val Glu Val Phe Cys Lys Lys His Gly Leu Val Tyr Glu Asp
485 490 495

Val Ser Ile Ala Thr Gly Thr Cys Lys Val Leu Lys Ala Leu Lys Glu
500 505 510

Val Ala Glu Ala Ala Ala Glu Gln His Ala Thr Thr Ser
515 520 525

INTERNATIONAL SEARCH REPORT

Int. Application No

PCT/EP 00/06223

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/82 C12N9/02 C12N15/53 C12P7/64 C11C3/00
 A01H5/00 A01H13/00 A01H15/00 A23L1/30 A23K1/16
 A61K35/78

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, EMBASE, BIOSIS, MEDLINE, SCISEARCH, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	SPERLING, P. ET AL.: "A bifunctional delta-6-fatty acyl acetylenase/desaturase from the moss <i>Ceratodon purpureus</i> " EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 267, June 2000 (2000-06), pages 3801-3811, XP000941309 the whole document	1-4,7-11
X	GIRKE, T. ET AL.: "Identification of a novel delta-6-acyl-group desaturase by targeted gene disruption in <i>Physcomitrella patens</i> " THE PLANT JOURNAL, vol. 15, no. 1, July 1998 (1998-07), pages 39-48, XP000881712 cited in the application	1-4,7-11
Y	the whole document	5,6
	-/-	



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *Z* document member of the same patent family

Date of the actual completion of the international search

9 November 2000

Date of mailing of the international search report

24/11/2000

Name and mailing address of the ISA

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 NL - 2280 HV Rijswijk
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
 Fax: (+31-70) 340-3016

Authorized officer

Donath, C

INTERNATIONAL SEARCH REPORT

Int. l. Application No

PCT/EP 00/06223

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 46764 A (CALGENE LLC) 22 November 1998 (1998-11-22) cited in the application	11, 12
Y	page 5, line 27 -page 6, line 17 page 8, line 19 -page 36, line 27; examples 6-8, 13, 14, 16	1-10
X	WO 99 27111 A (UNIVERSITY OF BRISTOL) 3 June 1999 (1999-06-03) cited in the application	11
Y	page 4, line 7 -page 9, line 28; examples 1, 2	1-10
X	SAYANOVA, O. ET AL.: "Expression of a borage desaturase cDNA containing an N-terminal cytochrome b5 domain results in the accumulation of high levels of delta-6-desaturated fatty acids in transgenic tobacco" PROC.NATL.ACAD.SCI.USA, vol. 94, April 1997 (1997-04), pages 4211-4216, XP002099447 cited in the application	11
Y	the whole document	1-10
X	WO 96 21022 A (RHONE-POULENC AGROCHIMIE) 11 July 1996 (1996-07-11) cited in the application	11
Y	page 3, line 3 - line 23 page 5, line 16 -page 19, line 24; examples 6, 13, 14	1-10

INTERNATIONAL SEARCH REPORT

information on patent family members

Inter Application No

PCT/EP 00/06223

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9846764 A	22-10-1998	US 5972664 A	26-10-1999
		US 6075183 A	13-06-2000
		US 5968809 A	19-10-1999
		US 6051754 A	18-04-2000
		AU 720677 B	08-06-2000
		AU 7114798 A	11-11-1998
		AU 720725 B	08-06-2000
		AU 7114898 A	11-11-1998
		BG 103796 A	31-05-2000
		BG 103798 A	31-05-2000
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		BR 9809083 A	01-08-2000
		CN 1253587 T	17-05-2000
		CN 1253588 T	17-05-2000
		EP 0996732 A	03-05-2000
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		NO 994926 A	30-11-1999
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		PL 336077 A	05-06-2000
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		CN 1252099 T	03-05-2000
		EP 0975766 A	02-02-2000
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		PL 336143 A	05-06-2000
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WO 9927111 A	03-06-1999	AU 1249799 A	15-06-1999
		EP 1032682 A	06-09-2000
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WO 9621022 A	11-07-1996	US 5614393 A	25-03-1997
		AU 707061 B	01-07-1999
		AU 4673596 A	24-07-1996
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		EP 0801680 A	22-10-1997
		JP 10511848 T	17-11-1998
		US 5789220 A	04-08-1998

INTERNATIONALER RESEARCHBERICHT

Internationales Aktenzeichen

PCT/EP 00/06223

A. KLASSIFIZIERUNG DES ANMELDUNGSGEGENSTANDES

IPK 7 C12N15/82 C12N9/02 C12N15/53 C12P7/64 C11C3/00
 A01H5/00 A01H13/00 A01H15/00 A23L1/30 A23K1/16
 A61K35/78

Nach der Internationalen Patentklassifikation (IPK) oder nach der nationalen Klassifikation und der IPK

B. RECHERCHIERTE GEBIETE

Recherchiert Mindestprüfstoff (Klassifikationssystem und Klassifikationssymbole)

IPK 7 C12N C12P

Recherchierte aber nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Gebiete fallen

Während der internationalen Recherche konsultierte elektronische Datenbank (Name der Datenbank und evtl. verwendete Suchbegriffe)

EPO-Internal, WPI Data, PAJ, EMBASE, BIOSIS, MEDLINE, SCISEARCH, STRAND

C. ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie*	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
P,X	SPERLING, P. ET AL.: "A bifunctional delta-6-fatty acyl acetylenase/desaturase from the moss <i>Ceratodon purpureus</i> " EUROPEAN JOURNAL OF BIOCHEMISTRY, Bd. 267, Juni 2000 (2000-06), Seiten 3801-3811, XP000941309 das ganze Dokument	1-4,7-11
X	GIRKE, T. ET AL.: "Identification of a novel delta-6-acyl-group desaturase by targeted gene disruption in <i>Physcomitrella patens</i> " THE PLANT JOURNAL, Bd. 15, Nr. 1, Juli 1998 (1998-07), Seiten 39-48, XP000881712 in der Anmeldung erwähnt	1-4,7-11
Y	das ganze Dokument	5,6

☒ Weitere Veröffentlichungen sind der Fortsetzung von Feld C zu entnehmen

☒ Siehe Anhang Patentfamilie

* Besondere Kategorien von angegebenen Veröffentlichungen :

A Veröffentlichung, die den allgemeinen Stand der Technik definiert, aber nicht als besonders bedeutsam anzusehen ist

E älteres Dokument, das jedoch erst am oder nach dem internationalen Anmeldedatum veröffentlicht worden ist

L Veröffentlichung, die geeignet ist, einen Prioritätsanspruch zweifelhaft erscheinen zu lassen, oder durch die das Veröffentlichungsdatum einer anderen im Recherchenbericht genannten Veröffentlichung belegt werden soll oder die aus einem anderen besonderen Grund angegeben ist (wie ausgeführt)

O Veröffentlichung, die sich auf eine mündliche Offenbarung, eine Benutzung, eine Ausstellung oder andere Maßnahmen bezieht

P Veröffentlichung, die vor dem internationalen Anmeldedatum, aber nach dem beanspruchten Prioritätsdatum veröffentlicht worden ist

T Spätere Veröffentlichung, die nach dem internationalen Anmeldedatum oder dem Prioritätsdatum veröffentlicht worden ist und mit der Anmeldung nicht kollidiert, sondern nur zum Verständnis des der Erfindung zugrundeliegenden Prinzips oder der ihr zugrundeliegenden Theorie angegeben ist

X Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann allein aufgrund dieser Veröffentlichung nicht als neu oder auf erfinderscher Tätigkeit beruhend betrachtet werden

Y Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann nicht als auf erfinderscher Tätigkeit beruhend betrachtet werden, wenn die Veröffentlichung mit einer oder mehreren anderen Veröffentlichungen dieser Kategorie in Verbindung gebracht wird und diese Verbindung für einen Fachmann naheliegend ist

Z Veröffentlichung, die Mitglied derselben Patentfamilie ist

Datum des Abschlusses der internationalen Recherche

9. November 2000

Absendedatum des internationalen Recherchenberichts

24/11/2000

Name und Postanschrift der internationalen Recherchenbehörde

Europäisches Patentamt, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
 Fax: (+31-70) 340-3016

Bevollmächtigter Beauftragter

Donath, C

INTERNATIONALER RECHERCHENBERICHT

Inte. onales Aktenzeichen

PCT/EP 00/06223

C.(Fortsetzung) ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
X	WO 98 46764 A (CALGENE LLC) 22. November 1998 (1998-11-22) in der Anmeldung erwähnt	11,12
Y	Seite 5, Zeile 27 -Seite 6, Zeile 17 Seite 8, Zeile 19 -Seite 36, Zeile 27; Beispiele 6-8,13,14,16 ---	1-10
X	WO 99 27111 A (UNIVERSITY OF BRISTOL) 3. Juni 1999 (1999-06-03) in der Anmeldung erwähnt	11
Y	Seite 4, Zeile 7 -Seite 9, Zeile 28; Beispiele 1,2 ---	1-10
X	SAYANOVA, O. ET AL.: "Expression of a borage desaturase cDNA containing an N-terminal cytochrome b5 domain results in the accumulation of high levels of delta-6-desaturated fatty acids in transgenic tobacco" PROC.NATL.ACAD.SCI.USA, Bd. 94, April 1997 (1997-04), Seiten 4211-4216, XP002099447 in der Anmeldung erwähnt	11
Y	das ganze Dokument ---	1-10
X	WO 96 21022 A (RHONE-POULENC AGROCHIMIE) 11. Juli 1996 (1996-07-11) in der Anmeldung erwähnt	11
Y	Seite 3, Zeile 3 - Zeile 23 Seite 5, Zeile 16 -Seite 19, Zeile 24; Beispiele 6,13,14 -----	1-10

INTERNATIONALER RESEARCHBERICHT

Angaben zu Veröffentlichungen, die zur selben Patentfamilie gehören

Inter. Aktenzeichen

PCT/EP 00/06223

Im Recherchenbericht angeführtes Patentdokument	Datum der Veröffentlichung	Mitglied(er) der Patentfamilie	Datum der Veröffentlichung
WO 9846764 A	22-10-1998	US 5972664 A	26-10-1999
		US 6075183 A	13-06-2000
		US 5968809 A	19-10-1999
		US 6051754 A	18-04-2000
		AU 720677 B	08-06-2000
		AU 7114798 A	11-11-1998
		AU 720725 B	08-06-2000
		AU 7114898 A	11-11-1998
		BG 103796 A	31-05-2000
		BG 103798 A	31-05-2000
		BR 9808506 A	23-05-2000
		BR 9809083 A	01-08-2000
		CN 1253587 T	17-05-2000
		CN 1253588 T	17-05-2000
		EP 0996732 A	03-05-2000
		EP 1007691 A	14-06-2000
		NO 994924 A	30-11-1999
		NO 994926 A	30-11-1999
		PL 336067 A	05-06-2000
		PL 336077 A	05-06-2000
		WO 9846765 A	22-10-1998
		AU 6961698 A	11-11-1998
		BG 103797 A	28-04-2000
		BR 9808507 A	23-05-2000
		CN 1252099 T	03-05-2000
		EP 0975766 A	02-02-2000
		NO 994925 A	30-11-1999
		PL 336143 A	05-06-2000
		WO 9846763 A	22-10-1998
WO 9927111 A	03-06-1999	AU 1249799 A	15-06-1999
		EP 1032682 A	06-09-2000
		ZA 9810716 A	16-06-1999
WO 9621022 A	11-07-1996	US 5614393 A	25-03-1997
		AU 707061 B	01-07-1999
		AU 4673596 A	24-07-1996
		BR 9510411 A	19-05-1998
		CA 2207906 A	11-07-1996
		CN 1177379 A	25-03-1998
		EP 0801680 A	22-10-1997
		JP 10511848 T	17-11-1998
		US 5789220 A	04-08-1998

VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS

PCT

INTERNATIONALER VORLÄUFIGER PRÜFUNGSBERICHT

(Artikel 36 und Regel 70 PCT)

Aktenzeichen des Anmelders oder Anwalts 0050/050461	WEITERES VORGEHEN siehe Mitteilung über die Übersendung des internationalen vorläufigen Prüfungsberichts (Formblatt PCT/IPEA/416)	
Internationales Aktenzeichen PCT/EP00/06223	Internationales Anmeldedatum (Tag/Monat/Jahr) 04/07/2000	Prioritätsdatum (Tag/Monat/Tag) 06/07/1999
Internationale Patentklassifikation (IPK) oder nationale Klassifikation und IPK C12N15/82		
Anmelder BASF PLANT SCIENCE GMBH et al.		



1. Dieser internationale vorläufige Prüfungsbericht wurde von der mit der internationalen vorläufigen Prüfung beauftragten Behörde erstellt und wird dem Anmelder gemäß Artikel 36 übermittelt.
2. Dieser BERICHT umfaßt insgesamt 6 Blätter einschließlich dieses Deckblatts.

☐ Außerdem liegen dem Bericht ANLAGEN bei; dabei handelt es sich um Blätter mit Beschreibungen, Ansprüchen und/oder Zeichnungen, die geändert wurden und diesem Bericht zugrunde liegen, und/oder Blätter mit vor dieser Behörde vorgenommenen Berichtigungen (siehe Regel 70.16 und Abschnitt 607 der Verwaltungsrichtlinien zum PCT).

Diese Anlagen umfassen insgesamt Blätter.

3. Dieser Bericht enthält Angaben zu folgenden Punkten:

- I ☒ Grundlage des Berichts
- II ☐ Priorität
- III ☐ Keine Erstellung eines Gutachtens über Neuheit, erfinderische Tätigkeit und gewerbliche Anwendbarkeit
- IV ☐ Mangelnde Einheitlichkeit der Erfindung
- V ☒ Begründete Feststellung nach Artikel 35(2) hinsichtlich der Neuheit, der erfinderischen Tätigkeit und der gewerblichen Anwendbarkeit; Unterlagen und Erklärungen zur Stützung dieser Feststellung
- VI ☐ Bestimmte angeführte Unterlagen
- VII ☐ Bestimmte Mängel der internationalen Anmeldung
- VIII ☒ Bestimmte Bemerkungen zur internationalen Anmeldung

Datum der Einreichung des Antrags 11/12/2000	Datum der Fertigstellung dieses Berichts 19.10.2001
Name und Postanschrift der mit der internationalen vorläufigen Prüfung beauftragten Behörde:  Europäisches Patentamt D-80298 München Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Bevollmächtigter Bediensteter Donath, C Tel. Nr. +49 89 2399 8710 



INTERNATIONALER VORLÄUFIGER PRÜFUNGSBERICHT

Internationales Aktenzeichen PCT/EP00/06223

I. Grundlage des Berichts

1. Hinsichtlich der **Bestandteile** der internationalen Anmeldung (*Ersatzblätter, die dem Anmeldeamt auf eine Aufforderung nach Artikel 14 hin vorgelegt wurden, gelten im Rahmen dieses Berichts als "ursprünglich eingereicht" und sind ihm nicht beigelegt, weil sie keine Änderungen enthalten (Regeln 70.16 und 70.17)*):
Beschreibung, Seiten:

1-34 ursprüngliche Fassung

Patentansprüche, Nr.:

1-12 ursprüngliche Fassung

Sequenzprotokoll in der Beschreibung, Seiten:

1-6, in der ursprünglich eingereichten Fassung.

2. Hinsichtlich der **Sprache**: Alle vorstehend genannten Bestandteile standen der Behörde in der Sprache, in der die internationale Anmeldung eingereicht worden ist, zur Verfügung oder wurden in dieser eingereicht, sofern unter diesem Punkt nichts anderes angegeben ist.

Die Bestandteile standen der Behörde in der Sprache: zur Verfügung bzw. wurden in dieser Sprache eingereicht; dabei handelt es sich um

- ☐ die Sprache der Übersetzung, die für die Zwecke der internationalen Recherche eingereicht worden ist (nach Regel 23.1(b)).
- ☐ die Veröffentlichungssprache der internationalen Anmeldung (nach Regel 48.3(b)).
- ☐ die Sprache der Übersetzung, die für die Zwecke der internationalen vorläufigen Prüfung eingereicht worden ist (nach Regel 55.2 und/oder 55.3).

3. Hinsichtlich der in der internationalen Anmeldung offenbarten **Nucleotid- und/oder Aminosäuresequenz** ist die internationale vorläufige Prüfung auf der Grundlage des Sequenzprotokolls durchgeführt worden, das:

- ☒ in der internationalen Anmeldung in schriftlicher Form enthalten ist.
- ☒ zusammen mit der internationalen Anmeldung in computerlesbarer Form eingereicht worden ist.
- ☐ bei der Behörde nachträglich in schriftlicher Form eingereicht worden ist.
- ☐ bei der Behörde nachträglich in computerlesbarer Form eingereicht worden ist.
- ☐ Die Erklärung, daß das nachträglich eingereichte schriftliche Sequenzprotokoll nicht über den Offenbarungsgehalt der internationalen Anmeldung im Anmeldezeitpunkt hinausgeht, wurde vorgelegt.
- ☐ Die Erklärung, daß die in computerlesbarer Form erfassten Informationen dem schriftlichen Sequenzprotokoll entsprechen, wurde vorgelegt.

4. Aufgrund der Änderungen sind folgende Unterlagen fortgefallen:

☐ Beschreibung, Seiten:



INTERNATIONALER VORLÄUFIGER PRÜFUNGSBERICHT

Internationales Aktenzeichen PCT/EP00/06223

- ☐ Ansprüche, Nr.:
☐ Zeichnungen, Blatt:

5. ☐ Dieser Bericht ist ohne Berücksichtigung (von einigen) der Änderungen erstellt worden, da diese aus den angegebenen Gründen nach Auffassung der Behörde über den Offenbarungsgehalt in der ursprünglich eingereichten Fassung hinausgehen (Regel 70.2(c)).

(Auf Ersatzblätter, die solche Änderungen enthalten, ist unter Punkt 1 hinzuweisen; sie sind diesem Bericht beizufügen).

6. Etwaige zusätzliche Bemerkungen:

V. Begründete Feststellung nach Artikel 35(2) hinsichtlich der Neuheit, der erfinderischen Tätigkeit und der gewerblichen Anwendbarkeit; Unterlagen und Erklärungen zur Stützung dieser Feststellung

1. Feststellung

Neuheit (N)	Ja: Ansprüche	5,6
	Nein: Ansprüche	1-4,7-12
Erfinderische Tätigkeit (ET)	Ja: Ansprüche	
	Nein: Ansprüche	1-12
Gewerbliche Anwendbarkeit (GA)	Ja: Ansprüche	1-12
	Nein: Ansprüche	

2. Unterlagen und Erklärungen siehe Beiblatt

VIII. Bestimmte Bemerkungen zur internationalen Anmeldung

Zur Klarheit der Patentansprüche, der Beschreibung und der Zeichnungen oder zu der Frage, ob die Ansprüche in vollem Umfang durch die Beschreibung gestützt werden, ist folgendes zu bemerken:
siehe Beiblatt



Ad section V.:

1. Auf folgende Dokumente wird in diesem Bescheid Bezug genommen:

D1 The plant Journal 15(1), 39-48, 1998

D2 WO-A-98/46764

D3 WO-A-96/21022

2. Die vorliegende Internationale Anmeldung betrifft ein Verfahren zur Herstellung von ungesättigten Fettsäuren. Es werden transgene Organismen hergestellt (vorzugsweise Pflanzen, Algen oder Pilze), die aufgrund der Expression einer delta-6-Desaturase aus Moos einen erhöhten Gehalt an Fettsäuren, Ölen oder Lipiden mit delta-6-Doppelbindungen aufweisen. Desweiteren betrifft die Internationale Anmeldung die für das obige Verfahren hergestellten transgenen Organismen, die durch das Verfahren hergestellten Öle, Lipide oder Fettsäuren, sowie deren Verwendung in Futtermitteln, Nahrungsmitteln, Kosmetika oder Pharmazeutika.

Im Hinblick auf die im Internationalen Recherchenbericht zitierten Dokumente können nur die Ansprüche 5 und 6 der vorliegenden Internationalen Anmeldung als neu betrachtet werden (Artikel 33(2) PCT).

- 2.1 D1 offenbart die Isolierung und Klonierung einer cDNA sowie der dazu korrespondierenden genomischen DNA-Sequenz aus dem Moos *Physcomitrella patens*. Das von dieser DNA kodierte Protein wurde als eine delta-6-Desaturase identifiziert. Durch Expression des Proteins in *S.cerevisiae* sowie durch Analyse der aus diesem transgenen Organismus gewonnenen Fettsäuren konnte bestätigt werden, daß die klonierte DNA für eine delta-6-Desaturase kodiert. Sowohl die Nukleotid-Sequenz als auch die Aminosäure-Sequenz der in D1 isolierten DNA bzw. des korrespondierenden Proteins weisen eine 100 %ige Identität über die gesamte Länge mit der in der vorliegenden Internationalen Anmeldung offenbarten Sequenz SEQ ID NO:1 bzw. SEQ ID NO:2 auf (s.D1,S.44-47, 'Functional expression of PPDES6 in *Saccharomyces cerevisiae*', 'Discussion', 'Expression in *S.cerevisiae*', 'Lipid analysis' and Fig.1).

Das obige Dokument ist daher neuheitsschädlich für den Gegenstand der



Ansprüche 1-4 und 7-11.

- 2.2 D2 beschreibt eine Methode zur Herstellung von mehrfach ungesättigten langkettigen Fettsäuren in Pflanzen. Expressionskonstrukte enthaltend DNA-Sequenzen kodierend für eine delta-6-, delta-5- oder delta-12-Desaturase wurden zunächst zur Herstellung dieser transgenen Pflanzen verwendet. Es wurde gezeigt, daß eine Expression dieser Desaturasen in den Pflanzen die Herstellung von großen Mengen an mehrfach ungesättigten Fettsäuren ermöglicht, und auf diese Weise zu einer Veränderung des Fettsäure-Profils dieser Pflanzen führt. Diese Manipulation des Fettsäure-Profils erlaubt nunmehr die Herstellung von kommerziell nutzbaren Mengen an Pflanzenölen sowie deren Verwendung als Pharmazeutika, Nahrungsmittel etc. (s.D2, S.5, Zeile 27 - S.6, Zeile 17, S.8, Zeile 19 - S.36, Zeile 27, Beispiele 6-8,13,14,16).

In Hinblick auf D2 ist der Gegenstand der Ansprüche 11 und 12 daher nicht neu.

- 2.3 D3 offenbart die Klonierung einer DNA kodierend für eine delta-6-Desaturase aus dem Cyanobakterium Synechocystis sowie einer cDNA kodierend für eine delta-6-Desaturase aus Borretsch. Diese DNA-Sequenzen wurden in verschiedenen Organismen, wie z.B. in Tabakpflanzen, exprimiert, und es wurde gezeigt, daß in den transgenen Organismen mittels dieser Sequenzen ungesättigte Fettsäuren, wie z.B. GLA, hergestellt wurden (s.D3, S.3, Zeilen 3-23, S.5. Zeile 16 - S.19, Zeile 24, Beispiele 6,13,14, Ansprüche 11-18).

Das obige Dokument ist daher neuheitsschädlich für den Gegenstand des Anspruches 11.

3. Zur Beurteilung eines erfinderischen Schrittes der Ansprüche 5 und 6 der vorliegenden Internationalen Anmeldung wird ebenfalls D1 als der nächstliegende Stand der Technik herangezogen.
Diese Ansprüche betreffen die Verwendung einer transgenen Alge oder Pflanze, insbesondere einer Ölfuchtpflanze im Verfahren zur Herstellung von ungesättigten Fettsäuren.
Diese abhängigen Ansprüche scheinen keine zusätzlichen Merkmale zu enthalten, welche in Kombination mit den Merkmalen der Ansprüche auf die sie



sich beziehen, einen erfinderischen Schritt beinhalten. Die Verwendung transgener Pflanzen, bzw. Ölfruchtpflanzen, in einem Verfahren zur Herstellung von ungesättigten Fettsäuren ist bereits aus D2 oder D3 bekannt.

Der Gegenstand der Ansprüche 5 und 6 beruht daher nicht auf einer nach Artikel 33(3) PCT erforderlichen erfinderischen Tätigkeit.

Ad section VIII.:

1. Den Ansprüchen 1,4 und 7-9 mangelt es an Klarheit aufgrund der Ausdrücke "Organismus" und "Tiere". Die Beschreibung der vorliegenden internationalen Anmeldung nimmt nur Bezug auf tierische Zellen, nicht jedoch auf Tiere als solche. Desweiteren ist es absolut notwendig klarzustellen, daß der Mensch **nicht** unter die Begriffe "Organismus" und "Tiere" fällt.



11-11-77

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(12) NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES
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SN, TD, TG).

Veröffentlicht:

- Mit internationalem Recherchenbericht.
- Vor Ablauf der für Änderungen der Ansprüche geltenden
Frist; Veröffentlichung wird wiederholt, falls Änderungen
eintreffen.

Zur Erklärung der Zweibuchstaben-Codes, und der anderen
Abkürzungen wird auf die Erklärungen ("Guidance Notes on
Codes and Abbreviations") am Anfang jeder regulären Ausgabe
der PCT-Gazette verwiesen.

(54) Title: PLANTS EXPRESSING Δ 6-DESATURASE GENES AND OILS FROM THESE PLANTS CONTAINING PUFAS
AND METHOD FOR PRODUCING UNSATURATED FATTY ACIDS

(54) Bezeichnung: Δ 6-DESATURASEGENE EXPRIMIERENDE PFLANZEN UND PUFAS ENTHALTENDE ÖLE AUS DIE-
SEN PFLANZEN UND EIN VERFAHREN ZUR HERSTELLUNG UNGESÄTTIGTER FETTSÄUREN

(57) Abstract: The invention relates to an improved method for producing unsaturated fatty acids and to a method for producing
triglycerides with an increased unsaturated fatty acid content. The invention also relates to the production of a transgenic organism,
preferably a transgenic plant or a transgenic micro-organism, containing increased quantities of unsaturated fatty acids, oils or lipids
with Δ 6-double bonds as a result of the expression of a Δ -6-desaturase, from moss. The invention also relates to transgenic organ-
isms containing a Δ 6-desaturase gene, and to the use of the unsaturated fatty acids or triglycerides with an increased unsaturated
fatty acid content produced in the method.

(57) Zusammenfassung: Die vorliegende Erfindung betrifft ein verbessertes Verfahren zur Herstellung von ungesättigten Fettsäuren
sowie ein Verfahren zur Herstellung von Triglyceriden mit einem erhöhten Gehalt an ungesättigten Fettsäuren. Die Erfindung
betrifft die Herstellung eines transgenen Organismus bevorzugt einer transgenen Pflanze oder eines transgenen Mikroorganismus
mit erhöhtem Gehalt an Fettsäuren, Ölen oder Lipiden mit Δ 6-Doppelbindungen aufgrund der Expression einer Δ -6-Desaturase aus
Moos. Ausserdem betrifft die Erfindung transgene Organismen, die ein Δ 6-Desaturasegen enthalten, sowie die Verwendung der im
Verfahren hergestellten ungesättigten Fettsäuren bzw. Triglyceride mit einem erhöhten Gehalt an ungesättigten Fettsäuren.

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$\Delta 6$ -Desaturasegene exprimierende Pflanzen und PUFAS enthaltende Öle aus diesen Pflanzen und ein Verfahren zur Herstellung ungesättigter Fettsäuren

5

Beschreibung

Die vorliegende Erfindung betrifft ein verbessertes Verfahren zur Herstellung von ungesättigten Fettsäuren sowie ein Verfahren
10 zur Herstellung von Triglyceriden mit einem erhöhten Gehalt an ungesättigten Fettsäuren. Die Erfindung betrifft die Herstellung eines transgenen Organismusses bevorzugt einer transgenen Pflanze oder eines transgenen Mikroorganismus mit erhöhtem Gehalt an Fettsäuren, Ölen oder Lipiden mit $\Delta 6$ -Doppelbindungen aufgrund
15 der Expression einer $\Delta 6$ -Desaturase aus Moos.

Außerdem betrifft die Erfindung transgene Organismen, die ein $\Delta 6$ -Desaturasegen enthalten, sowie die Verwendung der im Verfahren hergestellten ungesättigten Fettsäuren bzw. Triglyceride mit
20 einem erhöhten Gehalt an ungesättigten Fettsäuren.

Fettsäuren und Triglyceride haben eine Vielzahl von Anwendungen in der Lebensmittelindustrie, der Tierernährung, der Kosmetik und im Pharmabereich. Je nachdem ob es sich um freie gesättigte oder
25 ungesättigte Fettsäuren oder um Triglyceride mit einem erhöhten Gehalt an gesättigten oder ungesättigten Fettsäuren handelt, sind sie für die unterschiedlichsten Anwendungen geeignet, so werden beispielsweise mehrfach ungesättigte Fettsäuren Babynahrung zur Erhöhung des Nährwertes zugesetzt. Hauptsächlich werden die ver-
30 schiedenen Fettsäuren und Triglyceride aus Mikroorganismen wie Mortierella oder aus Öl-produzierenden Pflanzen wie Soja, Raps, Sonnenblume und weiteren gewonnen, wobei sie in der Regel in Form ihrer Triacylglyceride anfallen. Sie können aber auch aus Tieren wie Fischen gewonnen werden. Die freien Fettsäuren werden vor-
35 teilhaft durch Verseifung hergestellt.

Je nach Anwendungszweck sind Öle mit gesättigten oder ungesättigten Fettsäuren bevorzugt, so sind z.B. in der humanen Ernährung Lipide mit ungesättigten Fettsäuren speziell mehrfach ungesättig-
40 ten Fettsäuren bevorzugt, da sie einen positiven Einfluß auf den Cholesterinspiegel im Blut und damit auf die Möglichkeit einer Herzerkrankung haben. Auch eine positive Wirkung auf die Carcinogenese wird den ungesättigten Fettsäuren zugeschrieben. Sie sind außerdem wichtige Ausgangsstoffe für die Synthese von
45 Verbindungen, die wichtige biologische Vorgänge innerhalb des

Organismus steuern. Sie finden deshalb in verschiedenen diätischen Lebensmitteln oder Medikamenten Anwendung.

- Aufgrund ihrer positiven Eigenschaften hat es in der Vergangenheit nicht an Ansätzen gefehlt, Gene, die an der Synthese von Fettsäuren bzw. Triglyceriden beteiligt sind, für die Herstellung von Ölen in verschiedenen Organismen mit geändertem Gehalt an ungesättigten Fettsäuren verfügbar zu machen. So wird in WO 91/13972 und seinem US-Äquivalent eine $\Delta 9$ -Desaturase beschrieben. In WO 93/11245 wird eine $\Delta 15$ -Desaturase in WO 94/11516 wird eine $\Delta 12$ -Desaturase beansprucht. $\Delta 6$ -Desaturasen werden in Girke et al. (The Plant Journal, 15, 1998: 39-48), Napier et al. (Biochem. J., 330, 1998: 611-614), Murata et al. (Biosynthesis of γ -linolenic acid in cyanobacterium *Spirulina patensis*, pp 22-32, In: γ -linolenic acid, metabolism and its roles in nutrition and medicine, Huang, Y. and Milles, D.E. [eds.], AOC Press, Champaign, Illinois), Sayanova et al. (Proc. Natl. Acad. Sci. USA, 94, 1997: 4211-4216), WO 98/46764, Cho et al. (J. Biol. Chem., 274, 1999: 471-477), Aki et al. (Biochem. Biophys. Res. Commun., 255, 1999: 575-579), und Reddy et al. (Plant Mol. Biol., 27, 1993: 293-300) beschrieben. Weitere Desaturasen werden beispielsweise in EP-A-0 550 162, WO 94/18337, WO 97/30582, WO 97/21340, WO 95/18222, EP-A-0 794 250, Stukey et al., J. Biol. Chem., 265, 1990: 20144-20149, Wada et al., Nature 347, 1990: 200-203 oder Huang et al., Lipids 34, 1999: 649-659 beschrieben. Weitere $\Delta 6$ -Desaturasen werden in WO 93/06712, US 5,614,393, US5,614,393, WO 96/21022, WO00/21557 und WO 99/27111 beschrieben. Die biochemische Charakterisierung der verschiedenen Desaturasen ist jedoch bisher nur unzureichend erfolgt, da die Enzyme als membrangebundene Proteine nur sehr schwer zu isolieren und charakterisieren sind (McKeon et al., Methods in Enzymol. 71, 1981: 12141-12147, Wang et al., Plant Physiol. Biochem., 26, 1988: 777-792). In der Regel erfolgt die Charakterisierung membrangebundener Desaturasen durch Einbringung in einen geeigneten Organismus, der anschließend auf Enzymaktivität mittels Edukt- und Produktanalyse untersucht wird. Die Anwendung zur Produktion in transgenen Organismen beschrieben wie in WO 98/46763 WO98/46764, WO98/46765. Dabei wird auch die Expression verschiedener Desaturasen wie in WO99/64616 oder WO98/46776 und Bildung polyungesättigter Fettsäuren beschrieben und beansprucht. Bezüglich der Effektivität der Expression von Desaturasen und ihren Einfluß auf die Bildung polyungesättigter Fettsäuren ist anzumerken, daß durch Expression einer einzelnen Desaturase wie im vorgenannten Stand der Technik beschrieben lediglich geringe Gehalte an ungesättigten Fettsäuren beispielsweise an $\Delta 6$ ungesättigten Fettsäuren/Lipiden wie z.B. γ -Linolensäure erreicht wurden und werden.

Nach wie vor besteht daher ein großer Bedarf an neuen und besser geeigneten Genen, die für Enzyme codieren, die an der Biosynthese ungesättigter Fettsäuren beteiligt sind und es ermöglichen, diese in einem technischen Maßstab herzustellen. Weiterhin besteht

5 nach wie vor ein Bedarf an verbesserten Verfahren zur Gewinnung möglichst hoher Gehalte an polyungesättigten Fettsäuren.

Es bestand daher die Aufgabe ein Verfahren zur Herstellung von ungesättigten Fettsäuren unter Verwendung von Genen, die

10 beispielsweise für Desaturase-Enzyme codieren und die an der Synthese mehrfach ungesättigter Fettsäuren in den Samen einer Ölsaart beteiligt sind, bereitzustellen und so den Gehalt polyungesättigter Fettsäuren zu erhöhen. Diese Aufgabe wurde durch ein Verfahren zur Herstellung von ungesättigten Fettsäuren

15 gelöst, dadurch gekennzeichnet, daß mindestens eine isolierte Nukleinsäuresequenz, die für ein Polypeptid mit $\Delta 6$ -Desaturaseaktivität codiert, ausgewählt aus der Gruppe:

- a) einer Nukleinsäuresequenz mit der in SEQ ID NO: 1 dargestellten Sequenz,
- 20 b) Nukleinsäuresequenzen, die sich als Ergebnis des degenerierten genetischen Codes von der in SEQ ID NO: 1 ableiten
- 25 c) Derivate der in SEQ ID NO: 1 dargestellten Nukleinsäuresequenz, die für Polypeptide mit der in SEQ ID NO: 2 dargestellten Aminosäuresequenzen codieren und mindestens 50 % Homologie auf Aminosäureebene aufweisen, ohne daß die enzymatische Wirkung der Polypeptide wesentlich reduziert
- 30 ist,

in einen Organismus eingebracht wird, dieser Organismus angezogen wird, wobei der angezogene Organismus mindestens 1 Mol-% ungesättigte Fettsäuren bezogen auf den gesamten Fettsäuregehalt

35 im Organismus enthält.

Unter Anzucht des Organismus ist die Kultivierung von Pflanzen ebenso zu verstehen wie die Anzucht von eukaryontischen oder prokaryontischen Mikroorganismen wie Bakterien, Hefen, Pilzen,

40 Ciliaten, Algen, Cyanobakterien, tierischen oder pflanzlichen Zellen oder Zellverbänden oder die Anzucht von Tieren.

Die in den im erfindungsgemäßen Verfahren gewonnenen Organismen enthalten in der Regel ungesättigte Fettsäuren in Form von

45 gebundenen Fettsäuren, das heißt die ungesättigten Fettsäuren liegen überwiegend in Form ihrer Mono-, Di- oder Triglyceride, Glycolipide, Lipoproteine oder Phospholipide wie Öle oder Lipide

- oder sonstig als Ester oder Amide gebundenen Fettsäuren vor. Auch freie Fettsäuren sind in den Organismen in Form der freien Fettsäuren oder in Form ihrer Salze enthalten. Die freien oder gebundenen ungesättigten Fettsäuren enthalten vorteilhaft gegen-
- 5 über den Ausgangsorganismen einen erhöhten Gehalt an Fettsäuren mit $\Delta 6$ -Doppelbindungen wie vorteilhaft γ -Linolensäure. Die durch Anzucht im erfindungsgemäßen Verfahren gewonnenen Organismen und die in ihnen enthaltenen ungesättigten Fettsäuren können direkt beispielsweise zur Herstellung von pharmazeutischen
- 10 Zubereitungen, von Agrochemikalien, Futtermitteln oder Lebensmitteln verwendet werden oder aber nach Isolierung aus den Organismen. Dabei können alle Stufen der Aufreinigung der ungesättigten Fettsäuren verwendet werden, das heißt von Rohextrakten der Fettsäuren bis zu vollständig gereinigten Fettsäuren sind für
- 15 die Herstellung der vorgenannten Produkte geeignet. In einer vorteilhaften Ausführungsform können die gebundenen Fettsäuren aus beispielsweise den Ölen bzw. Lipiden beispielsweise über eine basische Hydrolyse z.B. mit NaOH oder KOH freigesetzt werden. Diese freien Fettsäuren können direkt im erhaltenen Gemisch oder
- 20 nach weiterer Aufreinigung zur Herstellung von pharmazeutischen Zubereitungen, von Agrochemikalien, Futtermitteln oder Lebensmitteln verwendet werden. Auch können die gebundenen oder freien Fettsäuren zur Umesterung oder Veresterung beispielsweise mit anderen Mono-, Di- oder Triglyceriden oder Glycerin verwendet
- 25 werden, um den Anteil an ungesättigten Fettsäuren in diesen Verbindungen beispielsweise in den Triglyceriden zu erhöhen.

- Ein weiterer erfindungsgemäßer Gegenstand ist ein Verfahren zur Herstellung von Triglyceriden mit einem erhöhten Gehalt an unge-
- 30 sättigten Fettsäuren, indem man Triglyceride mit gesättigten oder ungesättigten oder gesättigten und ungesättigten Fettsäuren mit mindestens einem der Protein, das durch die Sequenz SEQ ID NO: 2 codiert wird, inkubiert. Vorteilhaft wird das Verfahren in Gegenwart von Verbindungen durchgeführt, die Reduktionsäquivalente
- 35 aufnehmen oder abgeben können. Anschließend können die Fettsäuren aus den Triglyceriden freigesetzt werden.

- Die oben genannten Verfahren ermöglichen vorteilhaft die Synthese von Fettsäuren oder gebundenen Fettsäuren wie Triglyceriden mit
- 40 einem erhöhten Gehalt an Fettsäuren mit $\Delta 6$ -Doppelbindungen.

- Als Organismen für die genannten Verfahren seien beispielhaft Pflanzen wie Arabidopsis, Gerste, Weizen, Roggen, Hafer, Mais, Soja, Reis, Baumwolle, Zuckerrübe, Tee, Karotte, Paprika, Canola,
- 45 Sonnenblume, Flachs, Hanf, Kartoffel, Triticale, Tabak, Tomate, Raps, Kaffee, Tapioka, Maniok, Pfeilwurz, Tagetes, Alfalfa, Erdnuß, Rizinus, Kokosnuß, Ölpalme, Färbersaflor (Carthamus

- tinctorius), Salat und den verschiedenen Baum-, Nuß- und Weinspezies, oder Kakaobohne, Mikroorganismen wie Pilze *Mortierella*, *Saprolegnia* oder *Pythium*, Bakterien wie die Gattung *Escherichia*, Cyanobakterien, Algen oder Protozoen wie Dinoflagellaten wie
- 5 *Cryptocodium* genannt. Bevorzugt werden Organismen, die natürlicherweise Öle in größeren Mengen synthetisieren können wie Mikroorganismen wie Pilze wie *Mortierella alpina*, *Pythium insidiosum* oder Pflanzen wie Soja, Raps, Kokosnuß, Ölpalme, Canola, Färbersaflor (*Carthamus tinctorius*), Rizinus, Calendula,
- 10 Lein, Borretsch, Erdnuß, Kakaobohne oder Sonnenblume, besonders bevorzugt werden Soja, Raps oder Sonnenblume.

- Die in den Verfahren verwendeten Organismen werden je nach Wirtsorganismus in dem Fachmann bekannter Weise angezogen bzw.
- 15 gezüchtet. Mikroorganismen wie Bakterien, Pilze, Ciliaten, pflanzliche oder tierische Zellen werden in der Regel in einem flüssigen Medium, das eine Kohlenstoffquelle meist in Form von Zuckern, eine Stickstoffquelle meist in Form von organischen Stickstoffquellen wie Hefeextrakt oder Salzen wie Ammoniumsulfat,
- 20 Spurenelemente wie Eisen-, Mangan-, Magnesiumsalze und gegebenenfalls Vitamine enthält, bei Temperaturen zwischen 0°C und 100°C, bevorzugt zwischen 10°C bis 60°C unter je nach Organismus Sauerstoffbegasung oder in Abwesenheit von Sauerstoff angezogen. Dabei kann der pH der Nährflüssigkeit auf einen festen Wert gehalten
- 25 werden, das heißt der pH wird während der Anzucht reguliert oder der pH wird nicht reguliert und verändert sich während der Anzucht. Die Anzucht kann batch weise, semi batch weise oder kontinuierlich erfolgen. Nährstoffe können zu Beginn der Fermentation vorgelegt oder semikontinuierlich oder kontinuier-
- 30 lich nach gefüttert werden. Auch eine Anzucht auf festen Medien ist möglich.

- Pflanzen werden nach Transformation in der Regel zunächst regeneriert und anschließend wie üblich angezogen bzw. angebaut.
- 35 Dies kann im Gewächshaus oder im Freiland erfolgen.

- Aus den Organismen werden nach Anzucht die Lipide in üblicher Weise gewonnen. Hierzu können die Organismen nach Ernte zunächst aufgeschlossen werden oder direkt verwendet werden. Die Lipide
- 40 werden vorteilhaft mit geeigneten Lösungsmitteln wie apolare Lösungsmittel wie Hexan oder Ethanol, Isopropanol oder Gemischen wie Hexan/Isopropanol, Phenol/Chloroform/Isoamylalkohol bei Temperaturen zwischen 0°C bis 80°C, bevorzugt zwischen 20°C bis 50°C extrahiert. Die Biomasse wird in der Regel mit einem Über-
- 45 schuß an Lösungsmittel extrahiert beispielsweise einem Überschuß von Lösungsmittel zu Biomasse von 1:4. Das Lösungsmittel wird anschließend beispielsweise über eine Destillation entfernt.

Die Extraktion kann auch mit superkritischem CO₂ erfolgen. Nach Extraktion kann die restliche Biomasse beispielsweise über Filtration entfernt werden.

- 5 Das so gewonnene Rohöl kann anschließend weiter aufgereinigt werden, beispielsweise in dem Trübungen über das Versetzen mit polaren Lösungsmittel wie Aceton oder Chloroform und anschließender Filtration oder Zentrifugation entfernt werden. Auch eine weitere Reinigung über chromatographische Verfahren, 10 Destillation oder Kristallisation ist möglich.

Zur Gewinnung der freien Fettsäuren aus den Triglyceriden werden diese in üblicher Weise, wie oben beschrieben, verseift.

- 15 Ein weiterer Gegenstand der Erfindung sind ungesättigte Fettsäuren sowie Triglyceride mit einem erhöhten Gehalt an ungesättigten Fettsäuren, die nach den oben genannten Verfahren hergestellt wurden, sowie deren Verwendung zur Herstellung von Nahrungsmitteln, Tierfutter, Kosmetika oder Pharmazeutika. Hierzu 20 werden diese den Nahrungsmitteln, dem Tierfutter, den Kosmetika oder Pharmazeutika in üblichen Mengen zugesetzt.

- Im erfindungsgemäßen Verfahren wurden durch Expression einer $\Delta 6$ -Desaturase aus Moos in Organismen wie Pilze, Bakterien, 25 Tieren oder Pflanzen, bevorzugt Pilzen, Bakterien und Pflanzen, besonders bevorzugt in Pflanzen, ganz besonders bevorzugt in Ölfruchtpflanzen wie Raps, Canola, Lein, Soja, Sonnenblume, Borretsch, Rizinus, Ölpalme, Färbersaflor (*Carthamus tinctorius*), Kokosnuß, Erdnuß oder Kakaobohne höhere Gehalte an ungesättigten 30 Fettsäuren wie γ -Linolensäure erhalten. Auch die Expression in Feldfrüchten, wie Mais, Weizen, Roggen, Hafer, Triticale, Reis, Gerste, Alfalfa, oder Buschpflanzen (Kaffee, Kakao, Tee) ist vorteilhaft. Durch die Expression eines Gens, das für eine $\Delta 6$ -Desaturase aus Moos codiert, in den oben genannten Organismen 35 können Gehalte an ungesättigten Fettsäuren in den Organismen von mindestens 1 Mol-%, bevorzugt mindestens 3 Mol-%, besonders bevorzugt mindestens 4 Mol-%, ganz besonders bevorzugt mindestens 5 Mol-% erreicht werden.

- 40 Unter Derivate(n) sind beispielsweise funktionelle Homologe der von SEQ ID NO: 1 codierten Enzyme oder deren enzymatischer Aktivität, das heißt Enzyme, die dieselben enzymatischen Reaktionen wie die von SEQ ID NO: 1 katalysieren, zu verstehen. Diese Gene ermöglichen ebenfalls eine vorteilhafte Herstellung 45 von ungesättigten Fettsäuren mit Doppelbindungen in $\Delta 6$ -Position. Unter ungesättigten Fettsäuren sind im folgenden doppelt oder mehrfach ungesättigte Fettsäuren, die Doppelbindungen aufweisen,

zu verstehen. Die Doppelbindungen können konjugiert oder nicht konjugiert sein. Die in SEQ ID NO: 1 genannte Sequenz codiert für ein Enzym, das eine $\Delta 6$ -Desaturase-Aktivität aufweist.

- 5 Das erfindungsgemäße Enzym $\Delta 6$ -Desaturase führt vorteilhaft in Fettsäurereste von Glycerolipiden eine *cis*-Doppelbindung in Position C₆-C₇ ein (siehe SEQ ID NO: 1). Das Enzym hat außerdem eine $\Delta 6$ -Desaturase-Aktivität, die vorteilhaft in Fettsäurereste von Glycerolipiden ausschließlich eine *cis*-Doppelbindung in
- 10 Position C₆-C₇ einführt. Diese Aktivität hat auch das Enzym mit der in SEQ ID NO: 1 genannten Sequenz, bei dem es sich um eine monofunktionelle $\Delta 6$ -Desaturase handelt.

- Die im erfindungsgemäßen Verfahren verwendeten Nukleinsäure-
- 15 sequenz(en) (für die Anmeldung soll der singular den plural umfassen und umgekehrt) oder Fragmente davon können vorteilhaft zur Isolierung weiterer genomischer Sequenzen über Homologie-screening verwendet werden.

- 20 Die genannten Derivate lassen sich beispielsweise aus anderen Organismen eukaryontischen Organismen wie Pflanzen wie speziell Moosen, Dinoflagellaten oder Pilze isolieren.

- Weiterhin sind unter Derivaten bzw. funktionellen Derivaten der
- 25 in SEQ ID NO: 1 genannten Sequenz beispielsweise Allelvarianten zu verstehen, die mindestens 50 % Homologie auf der abgeleiteten Aminosäureebene, vorteilhaft mindestens 70 % Homologie, bevorzugt mindestens 80 % Homologie, besonders bevorzugt mindestens 85 % Homologie, ganz besonders bevorzugt 90 % Homologie aufweisen.
- 30 Die Homologie wurde über den gesamten Aminosäurebereich berechnet. Es wurde das Programm PileUp, BESTFIT, GAP, TRANSLATE bzw. BACKTRANSLATE (= Bestandteil des Programmpaketes UWGGC, Wisconsin Package, Version 10.0-UNIX, January 1999, Genetics Computer Group, Inc., Deverux et al., Nucleic. Acid Res., 12,
- 35 1984: 387-395) verwendet (J. Mol. Evolution., 25, 351-360, 1987, Higgins et al., CABIOS, 5 1989: 151-153). Die von den genannten Nukleinsäuren abgeleitete Aminosäuresequenz ist Sequenz SEQ ID NO: 2 zu entnehmen. Unter Homologie ist Identität zu verstehen, das heißt die Aminosäuresequenzen sind zu mindestens
- 40 50 % identisch. Die erfindungsgemäßen Sequenzen sind auf Nukleinsäureebene mindestens 65 % homolog, bevorzugt mindestens 70 %, besonders bevorzugt 75 %, ganz besonders bevorzugt mindestens 80 %.

- 45 Allelvarianten umfassen insbesondere funktionelle Varianten, die durch Deletion, Insertion oder Substitution von Nukleotiden aus der in SEQ ID NO: 1 dargestellten Sequenz erhältlich sind, wobei

die enzymatische Aktivität der abgeleiteten synthetisierten Proteine erhalten bleibt.

Solche DNA-Sequenzen lassen sich ausgehend von der in

- 5 SEQ ID NO: 1 beschriebenen DNA-Sequenz oder Teilen dieser Sequenzen, beispielsweise mit üblichen Hybridisierungsverfahren oder der PCR-Technik aus anderen Eukaryonten wie beispielsweise den oben genannt isolieren. Diese DNA-Sequenzen hybridisieren unter Standardbedingungen mit den genannten Sequenzen. Zur
- 10 Hybridisierung werden vorteilhaft kurze Oligonukleotide beispielsweise der konservierten Bereiche, die über Vergleiche mit anderen Desaturasegenen in dem Fachmann bekannter Weise ermittelt werden können, verwendet. Vorteilhaft werden die Histidin-Box-Sequenzen verwendet. Es können aber auch längere Fragmente der
- 15 erfindungsgemäßen Nukleinsäuren oder die vollständigen Sequenzen für die Hybridisierung verwendet werden. Je nach der verwendeten Nukleinsäure: Oligonukleotid, längeres Fragment oder vollständige Sequenz oder je nachdem welche Nukleinsäureart DNA oder RNA für die Hybridisierung verwendet werden, variieren diese Standard-
- 20 bedingungen. So liegen beispielsweise die Schmelztemperaturen für DNA:DNA-Hybride ca. 10°C niedriger als die von DNA:RNA-Hybriden gleicher Länge.

Unter Standardbedingungen sind beispielsweise je nach Nuklein-

- 25 säure Temperaturen zwischen 42 und 58°C in einer wäßrigen Pufferlösung mit einer Konzentration zwischen 0,1 bis 5 x SSC (1 x SSC = 0,15 M NaCl, 15 mM Natriumcitrat, pH 7,2) oder zusätzlich in Gegenwart von 50 % Formamid wie beispielsweise 42°C in 5 x SSC, 50 % Formamid zu verstehen. Vorteilhafterweise liegen die
- 30 Hybridisierungsbedingungen für DNA:DNA-Hybride bei 0,1 x SSC und Temperaturen zwischen etwa 20°C bis 45°C, bevorzugt zwischen etwa 30°C bis 45°C. Für DNA:RNA-Hybride liegen die Hybridisierungsbedingungen vorteilhaft bei 0,1 x SSC und Temperaturen zwischen etwa 30°C bis 55°C, bevorzugt zwischen etwa 45°C bis 55°C. Diese
- 35 angegebenen Temperaturen für die Hybridisierung sind beispielhaft kalkulierte Schmelztemperaturwerte für eine Nukleinsäure mit einer Länge von ca. 100 Nukleotiden und einem G + C-Gehalt von 50 % in Abwesenheit von Formamid. Die experimentellen Bedingungen für die DNA-Hybridisierung sind in einschlägigen Lehrbüchern der
- 40 Genetik wie beispielsweise Sambrook et al., "Molecular Cloning", Cold Spring Harbor Laboratory, 1989, beschrieben und lassen sich nach dem Fachmann bekannten Formeln beispielsweise abhängig von der Länge der Nukleinsäuren, der Art der Hybride oder dem G + C-Gehalt berechnen. Weitere Informationen zur Hybridisierung kann
- 45 der Fachmann folgenden Lehrbüchern entnehmen: Ausubel et al. (eds), 1985, Current Protocols in Molecular Biology, John Wiley & Sons, New York; Hames and Higgins (eds), 1985, Nucleic Acids

Hybridization: A Practical Approach, IRL Press at Oxford University Press, Oxford; Brown (ed), 1991, Essential Molecular Biology: A Practical Approach, IRL Press at Oxford University Press, Oxford.

5

Weiterhin sind unter Derivaten Homologe der Sequenz SEQ ID No: 1 beispielsweise eukaryontische Homologe, verkürzte Sequenzen, Einzelstrang-DNA der codierenden und nichtcodierenden DNA-Sequenz oder RNA der codierenden und nichtcodierenden DNA-Sequenz zu

10 verstehen.

Außerdem sind unter Homologen der Sequenz SEQ ID NO: 1 Derivate wie beispielsweise Promotorvarianten zu verstehen. Diese Varianten können durch ein oder mehrere Nukleotidaustausche, durch

15 Insertion(en) und/oder Deletion(en) verändert sein, ohne daß aber die Funktionalität bzw. Wirksamkeit der Promotoren beeinträchtigt sind. Des weiteren können die Promotoren durch Veränderung ihrer Sequenz in ihrer Wirksamkeit erhöht oder komplett durch wirksamere Promotoren auch artfremder Organismen ausgetauscht werden.

20

Unter Derivaten sind auch vorteilhaft Varianten zu verstehen, deren Nukleotidsequenz im Bereich -1 bis -2000 vor dem Startcodon so verändert wurden, daß die Genexpression und/oder die Proteinexpression verändert, bevorzugt erhöht wird. Weiterhin sind unter

25 Derivaten auch Varianten zu verstehen, die am 3'-Ende verändert wurden.

Die Nukleinsäuresequenzen, die für eine $\Delta 6$ -Desaturase codiert, können synthetisch hergestellt oder natürlich gewonnen sein oder

30 eine Mischung aus synthetischen und natürlichen DNA-Bestandteilen enthalten, sowie aus verschiedenen heterologen $\Delta 6$ -Desaturase-Genabschnitten verschiedener Organismen bestehen. Im allgemeinen werden synthetische Nukleotid-Sequenzen mit Codons erzeugt, die von den entsprechenden Wirtsorganismen beispielsweise Pflanzen

35 bevorzugt werden. Dies führt in der Regel zu einer optimalen Expression der heterologen Gene. Diese von Pflanzen bevorzugten Codons können aus Codons mit der höchsten Proteinhäufigkeit bestimmt werden, die in den meisten interessanten Pflanzenspezies exprimiert werden. Ein Beispiel für *Corynebacterium glutamicum* ist gegeben in: Wada et al. (1992) *Nucleic Acids Res.* 20:2111-2118). Die Durchführung solcher Experimente sind mit Hilfe von Standardmethoden durchführbar und sind dem Fachmann auf dem Gebiet bekannt.

45 Funktionell äquivalente Sequenzen, die für das $\Delta 6$ -Desaturase-Gen codieren, sind solche Derivate der erfindungsgemäßen Sequenz, welche trotz abweichender Nukleotidsequenz noch die gewünschten

Funktionen, das heißt die enzymatische Aktivität der Proteine besitzen. Funktionelle Äquivalente umfassen somit natürlich vorkommende Varianten der hierin beschriebenen Sequenzen sowie künstliche, z.B. durch chemische Synthese erhaltene, an den

5 Codon-Gebrauch einer Pflanze angepaßte, künstliche Nukleotid-Sequenzen.

Außerdem sind artifizielle DNA-Sequenzen geeignet, solange sie, wie oben beschrieben, die gewünschte Eigenschaft beispielsweise

10 weise der Erhöhung des Gehaltes von $\Delta 6$ -Doppelbindungen in Fettsäuren, Ölen oder Lipiden in der Pflanze durch Überexpression des $\Delta 6$ -Desaturase-Gens in Kulturpflanzen vermitteln. Solche artifiziellen DNA-Sequenzen können beispielsweise durch Rückübersetzung mittels Molecular Modelling konstruierter Proteine,

15 die $\Delta 6$ -Desaturase-Aktivität aufweisen oder durch in vitro-Selektion ermittelt werden. Mögliche Techniken zur in vitro-Evolution von DNA zur Veränderung bzw. Verbesserung der DNA-Sequenzen sind beschrieben bei Patten, P.A. et al., Current Opinion in Biotechnology 8, 724-733 (1997) oder bei Moore, J.C.

20 et al., Journal of Molecular Biology 272, 336-347 (1997). Besonders geeignet sind codierende DNA-Sequenzen, die durch Rückübersetzung einer Polypeptidsequenz gemäß der für die Wirtspflanze spezifischen odon-Nutzung erhalten werden. Die spezifische Codon-Nutzung kann ein mit pflanzengenetischen

25 Methoden vertrauter Fachmann durch Computerauswertungen anderer, bekannter Gene der zu transformierenden Pflanze leicht ermitteln.

Als weitere geeignete äquivalente Nukleinsäure-Sequenzen sind zu nennen Sequenzen, welche für Fusionsproteine codieren, wobei

30 Bestandteil des Fusionsproteins ein $\Delta 6$ -Desaturase-Polypeptid oder ein funktionell äquivalenter Teil davon ist. Der zweite Teil des Fusionsproteins kann z.B. ein weiteres Polypeptid mit enzymatischer Aktivität sein oder eine antigene Polypeptidsequenz mit deren Hilfe ein Nachweis auf $\Delta 6$ -Desaturase-Expression mög-

35 lich ist (z.B. myc-tag oder his-tag). Bevorzugt handelt es sich dabei jedoch um eine regulative Proteinsequenz, wie z.B. ein Signalsequenz für das ER, das das $\Delta 6$ -Desaturase-Protein an den gewünschten Wirkort leitet.

Vorteilhaft können die $\Delta 6$ -Desaturase-Gene im erfindungsgemäßen Verfahren mit weiteren Genen der Fettsäurebiosynthese kombiniert werden. Beispiele für derartige Gene sind die Acetyltransferasen, weitere Desaturasen oder Elongasen ungesättigter oder gesättigter Fettsäuren wie in WO 00/12720 beschrieben. Für die in-vivo und

45 speziell in-vitro Synthese ist die Kombination mit z.B. NADH-Cytochrom B5 Reduktasen vorteilhaft, die Reduktionsäquivalente aufnehmen oder abgeben können.

11

Unter den im erfindungsgemäßen Verfahren verwendeten Proteine sind Proteine zu verstehen, die eine in der Sequenz SEQ ID NO: 2 dargestellte Aminosäuresequenz oder eine daraus durch Substitution, Inversion, Insertion oder Deletion von einem oder

- 5 mehreren Aminosäureresten erhältliche Sequenz enthalten, wobei die enzymatische Aktivität des in SEQ ID NO: 2 dargestellten Proteins erhalten bleibt bzw. nicht wesentlich reduziert wird. Unter nicht wesentlich reduziert sind alle Enzyme zu verstehen, die noch mindestens 10 %, bevorzugt 20 %, besonders bevorzugt
- 10 30 % der enzymatischen Aktivität des Ausgangsenzyms aufweisen. Dabei können beispielsweise bestimmte Aminosäuren durch solche mit ähnlichen physikochemischen Eigenschaften (Raumerfüllung, Basizität, Hydrophobizität etc.) ersetzt werden. Beispielsweise werden Argininreste gegen Lysinreste, Valinreste gegen Isoleucin-
- 15 reste oder Asparaginsäurereste gegen Glutaminsäurereste ausgetauscht. Es können aber auch ein oder mehrere Aminosäuren in ihrer Reihenfolge vertauscht, hinzugefügt oder entfernt werden, oder es können mehrere dieser Maßnahmen miteinander kombiniert werden.

20

Unter Derivaten sind auch funktionelle Äquivalente zu verstehen, die insbesondere auch natürliche oder künstliche Mutationen einer ursprünglich isolierten für $\Delta 6$ -Desaturase codierende Sequenz beinhalten, welche weiterhin die gewünschte Funktion zeigen, das

25 heißt das deren enzymatische Aktivität nicht wesentlich reduziert ist. Mutationen umfassen Substitutionen, Additionen, Deletionen, Vertauschungen oder Insertionen eines oder mehrerer Nukleotidreste. Somit werden beispielsweise auch solche Nukleotidsequenzen durch die vorliegende Erfindung mit umfaßt, welche man durch

- 30 Modifikation der $\Delta 6$ -Desaturase Nukleotidsequenz erhält. Ziel einer solchen Modifikation kann z.B. die weitere Eingrenzung der darin enthaltenen codierenden Sequenz oder z.B. auch die Einfügung weiterer Restriktionsenzym-Schnittstellen sein.
- 35 Funktionelle Äquivalente sind auch solche Varianten, deren Funktion, verglichen mit dem Ausgangsgen bzw. Genfragment, abgeschwächt (= nicht wesentlich reduziert) oder verstärkt ist (= Enzymaktivität ist stärker als die Aktivität des Ausgangsenzym, das heißt Aktivität ist höher als 100 %, bevorzugt höher
- 40 als 110 %, besonders bevorzugt höher als 130 %).

Die im erfindungsgemäßen Verfahren verwendeten oben genannten Nukleinsäuresequenzen werden vorteilhaft zum Einbringen in einen Wirtsorganismus in eine Expressionskassette inseriert.

- 45 Die Nukleinsäuresequenzen können jedoch auch direkt in den Wirtsorganismus eingebracht werden. Die Nukleinsäuresequenz kann dabei vorteilhaft beispielsweise eine DNA- oder cDNA-Sequenz sein.

12

Zur Insertion in eine Expressionskassette geeignete codierende Sequenzen sind beispielsweise solche, die für eine $\Delta 6$ -Desaturase mit den oben beschriebenen Sequenzen codieren und die dem Wirt die Fähigkeit zur Überproduktion von Fettsäuren, Ölen oder

- 5 Lipiden mit Doppelbindungen in $\Delta 6$ -Position verleihen. Diese Sequenzen können homologen oder heterologen Ursprungs sein.

Unter einer Expressionskassette (= Nukleinsäurekonstrukt oder -fragment) ist die in SEQ ID NO: 1 genannte Sequenz, die sich

- 10 als Ergebnis des genetischen Codes und/oder deren funktionellen oder nicht funktionellen Derivate zu verstehen, die mit einem oder mehreren Regulationssignalen vorteilhafterweise zur Erhöhung der Genexpression funktionell verknüpft wurden und welche die Expression der codierenden Sequenz in der Wirtszelle steuern.
- 15 Diese regulatorischen Sequenzen sollen die gezielte Expression der Gene und der Proteinexpression ermöglichen. Dies kann beispielsweise je nach Wirtsorganismus bedeuten, daß das Gen erst nach Induktion exprimiert und/oder überexprimiert wird, oder daß es sofort exprimiert und/oder überexprimiert wird. Beispielsweise
- 20 handelt es sich bei diesen regulatorischen Sequenzen um Sequenzen an die Induktoren oder Repressoren binden und so die Expression der Nukleinsäure regulieren. Zusätzlich zu diesen neuen Regulationssequenzen oder anstelle dieser Sequenzen kann die natürliche Regulation dieser Sequenzen vor den eigentlichen Struktur-
- 25 genen noch vorhanden sein und gegebenenfalls genetisch verändert worden sein, so daß die natürliche Regulation ausgeschaltet und die Expression der Gene erhöht wurde. Das Genkonstrukt kann aber auch einfacher aufgebaut sein, das heißt es wurden keine zusätzlichen Regulationssignale vor die Nukleinsäuresequenz oder dessen
- 30 Derivate inseriert und der natürliche Promotor mit seiner Regulation wurde nicht entfernt. Stattdessen wurde die natürliche Regulationssequenz so mutiert, daß keine Regulation mehr erfolgt und/oder die Genexpression gesteigert wird. Diese veränderten Promotoren können in Form von Teilsequenzen (= Promotor mit
- 35 Teilen der erfindungsgemäßen Nukleinsäuresequenzen) auch allein vor das natürliche Gen zur Steigerung der Aktivität gebracht werden. Das Genkonstrukt kann außerdem vorteilhafterweise auch eine oder mehrere sogenannte "enhancer Sequenzen" funktionell verknüpft mit dem Promotor enthalten, die eine erhöhte Expression
- 40 der Nukleinsäuresequenz ermöglichen. Auch am 3'-Ende der DNA-Sequenzen können zusätzliche vorteilhafte Sequenzen inseriert werden wie weitere regulatorische Elemente oder Terminatoren. Das $\Delta 6$ -Desaturase-Gen kann in einer oder mehreren Kopien in der Expressionskassette (= Genkonstrukt) enthalten sein. Auch
- 45 eventuell mit exprimierte Gene, die vorteilhaft an der Fettsäurebiosynthese beteiligt sind, können in einer oder mehreren Kopien in der Expressionskassette vorhanden sein.

13

Die regulatorischen Sequenzen bzw. Faktoren können dabei wie oben beschrieben vorzugsweise die Genexpression der eingeführten Gene positiv beeinflussen und dadurch erhöhen. So kann eine Verstärkung der regulatorischen Elemente vorteilhafterweise auf der

5 Transkriptionsebene erfolgen, indem starke Transkriptionssignale wie Promotoren und/oder "Enhancer" verwendet werden. Daneben ist aber auch eine Verstärkung der Translation möglich, indem beispielsweise die Stabilität der mRNA verbessert wird.

- 10 Als Promotoren in der Expressionskassette sind grundsätzlich alle Promotoren geeignet, die die Expression von Fremdgenen in Organismen vorteilhaft in Pflanzen oder Pilzen steuern können. Vorzugsweise verwendet man insbesondere einen pflanzlichen Promotor oder Promotoren, die beispielsweise aus einem Pflanzen-
- 15 virus entstammen. Vorteilhafte Regulationssequenzen für das erfindungsgemäße Verfahren sind beispielsweise in Promotoren wie *cos-*, *tac-*, *trp-*, *tet-*, *trp-tet-*, *lpp-*, *lac-*, *lpp-lac-*, *lacIq-*, *T7-*, *T5-*, *T3-*, *gal-*, *trc-*, *ara-*, *SP6-*, λ -*P_R*- oder im λ -*P_L*-Promotor enthalten, die vorteilhafterweise in gram-negativen Bakterien
- 20 Anwendung finden. Weitere vorteilhafte Regulationssequenzen sind beispielsweise in den gram-positiven Promotoren *amy* und *SPO2*, in den Hefe- oder Pilzpromotoren *ADC1*, *MF α* , *AC*, *P-60*, *CYC1*, *GAPDH*, *TEF*, *rp28*, *ADH* oder in den Pflanzenpromotoren wie *CaMV/35S* [Franck et al., Cell 21(1980) 285-294], *RUBISCO SSU*, *OCS*, *B33*,
- 25 *nos* (= Nopalin Synthase Promotor) oder im Ubiquitin-Promotor enthalten. Die Expressionskassette kann auch einen chemisch induzierbaren Promotor enthalten, durch den die Expression des exogenen $\Delta 6$ -Desaturase-Gens in den Organismen vorteilhaft in den Pflanzen zu einem bestimmten Zeitpunkt gesteuert werden kann.
- 30 Derartige vorteilhafte Pflanzenpromotoren sind beispielsweise der *PRP1*-Promotor [Ward et al., Plant. Mol. Biol. 22 (1993), 361-366], ein durch Benzensulfonamid-induzierbarer (EP 388186), ein durch Tetrazyklin-induzierbarer (Gatz et al., (1992) Plant J. 2,397-404), ein durch Salizylsäure induzierbarer Promotor
- 35 (WO 95/19443), ein durch Abscisinsäure-induzierbarer (EP335528) bzw. ein durch Ethanol- oder Cyclohexanon-induzierbarer (WO 93/21334) Promotor. Weitere Pflanzenpromotoren sind beispielsweise der Promotor der cytosolischen FBPase aus Kartoffel, der *ST-LSI* Promotor aus Kartoffel (Stockhaus et al., EMBO J.
- 40 8 (1989) 2445-245), der Promotor der Phosphoribosylpyrophosphat Amidotransferase aus *Glycine max* (siehe auch Genbank Accession Nummer U87999) oder ein Nodien-spezifischen Promotor wie in EP 249676 können vorteilhaft verwandt werden. Vorteilhaft sind insbesondere solche pflanzliche Promotoren, die die Expression in
- 45 Geweben oder Pflanzenteilen/-organen sicherstellen, in denen die Fettsäurebiosynthese bzw. dessen Vorstufen stattfindet wie beispielsweise im Endosperm oder im sich entwickelnden Embryo. Ins-

besondere zu nennen sind vorteilhafte Promotoren, die eine samen-spezifische Expression gewährleisten wie beispielsweise der USP-Promotor oder Derivate davon, der LEB4-Promotor, der Phaseolin-Promotor oder der Napin-Promotor. Der erfindungsgemäß aufgeführte
5 und besonders vorteilhafte USP-Promotor oder dessen Derivate vermitteln in der Samenentwicklung eine sehr früh Genexpression (Baeumlein et al., Mol Gen Genet, 1991, 225 (3): 459-67). Weitere vorteilhafte samenspezifische Promotoren, die für monokotyle und dikotyle Pflanzen verwendet werden können, sind die für Dikotyle
10 geeignete Promotoren wie ebenfalls beispielhaft ausgeführte Napingen-Promotor aus Raps (US5,608,152), der Oleosin-Promotor aus Arabidopsis (WO98/45461), der Phaseolin-Promotor aus Phaseolus vulgaris (US5,504,200), der Bce4-Promotor aus Brassica (WO91/13980) oder der Leguminosen B4-Promotor (LeB4, Baeumlein
15 et al., Plant J., 2, 2, 1992: 233 - 239) oder für Monokotyle geeignete Promotoren wie die Promotoren des lpt2- oder lpt1-Gens aus Gerste (WO95/15389 und WO95/23230) oder die Promotoren des Gersten Hordein-Gens, des Reis Glutelin-Gens, des Reis Oryzin-Gens, des Reis Prolamin-Gens, des Weizen Gliadin-
20 Gens, des Weizen Glutelin-Gens, des Mais Zein-Gens, des Hafer Glutelin-Gens, des Sorghum Kasirin-Gens oder des Roggen Secalin-Gens, die in WO99/16890 beschrieben werden.

Weiterhin sind insbesondere solche Promotoren bevorzugt, die
25 die Expression in Geweben oder Pflanzenteilen sicherstellen, in denen beispielsweise die Biosynthese von Fettsäuren, Ölen und Lipiden bzw. deren Vorstufen stattfindet. Insbesondere zu nennen sind Promotoren, die eine samenspezifische Expression gewährleisten. Zu nennen sind der Promotor des Napin-Gens aus Raps
30 (US 5,608,152), des USP-Promotor aus Vicia faba (USP=unbekanntes Samenprotein, Baeumlein et al., Mol Gen Genet, 1991, 225 (3): 459-67), des Oleosin-Gens aus Arabidopsis (WO98/45461), des Phaseolin-Promotors (US 5,504,200) oder der Promotor des Legumin B4-Gens (LeB4; Baeumlein et al., 1992, Plant Journal, 2 (2):
35 233-9). Weiterhin sind zu nennen Promotoren, wie der des lpt2 oder lpt1-Gens aus Gerste (WO95/15389 und WO95/23230), die in monokotylen Pflanzen samenspezifische Expression vermitteln.

In der Expressionskassette (= Genkonstrukt, Nukleinsäurekon-
40 strukt) können wie oben beschrieben noch weitere Gene, die in die Organismen eingebracht werden sollen, enthalten sein. Diese Gene können unter getrennter Regulation oder unter der gleichen Regulationsregion wie das $\Delta 6$ -Desaturase-Gen liegen. Bei diesen Genen handelt es sich beispielsweise um weitere Biosynthesegene
45 vorteilhaft der Fettsäurebiosynthese, die eine gesteigerte Synthese ermöglichen. Beispielsweise seien die Gene für die $\Delta 15$ -, $\Delta 12$ -, $\Delta 9$ -, $\Delta 5$ -, $\Delta 4$ -Desaturase, die verschiedenen Hydroxylasen,

15

die Acyl-ACP-Thioesterasen, β -Ketoacyl-Synthasen oder β -Ketoacyl-Reductasen genannt. Vorteilhaft werden die Desaturasegene im Nukleinsäurekonstrukt verwendet.

5 Prinzipiell können alle natürlichen Promotoren mit ihren Regulationssequenzen wie die oben genannten für die erfindungsgemäße Expressionskassette und das erfindungsgemäße Verfahren, wie unten beschrieben, verwendet werden. Darüberhinaus können auch synthetische Promotoren vorteilhaft verwendet werden.

10

Es können verschiedene DNA-Fragmente manipuliert werden, um eine Nukleotid-Sequenz zu erhalten, die zweckmäßigerweise in der korrekten Richtung gelesen wird und die mit einem korrekten Leseraster ausgestattet ist. Für die Verbindung der DNA-Fragmente

15 (= erfindungsgemäße Nukleinsäuren) miteinander können an die Fragmente Adaptoren oder Linker angesetzt werden.

Zweckmäßigerweise können die Promotor- und die Terminator-Regionen in Transkriptionsrichtung mit einem Linker oder Poly-

20 linker, der eine oder mehrere Restriktionsstellen für die Insertion dieser Sequenz enthält, versehen werden. In der Regel hat der Linker 1 bis 10, meistens 1 bis 8, vorzugsweise 2 bis 6 Restriktionsstellen. Im allgemeinen hat der Linker innerhalb der regulatorischen Bereiche eine Größe von weniger als 100 bp, häufig weniger als 60 bp, mindestens jedoch 5 bp. Der Promotor kann sowohl nativ bzw. homolog als auch fremdartig bzw. heterolog zum Wirtsorganismus beispielsweise zur Wirtspflanze sein. Die Expressionskassette beinhaltet in der 5'-3'-Transkriptionsrichtung den Promotor, eine DNA-Sequenz, die für ein im er-

30 findungsgemäßen Verfahren verwendetes $\Delta 6$ -Desaturase-Gen codiert und eine Region für die transkriptionale Termination. Verschiedene Terminationsbereiche sind gegeneinander beliebig austauschbar.

35 Ferner können Manipulationen, die passende Restriktionsschnittstellen bereitstellen oder die überflüssige DNA oder Restriktionsschnittstellen entfernen, eingesetzt werden. Wo Insertionen, Deletionen oder Substitutionen wie z.B. Transitionen und Transversionen in Frage kommen, können *in vitro*-Mutagenese, -primer-repair-, Restriktion oder Ligation verwendet werden. Bei geeigneten Manipulationen, wie z.B. Restriktion, -chewing-back- oder Auffüllen von Überhängen für -bluntends-, können komplementäre Enden der Fragmente für die Ligation zur Verfügung gestellt werden.

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16

Von Bedeutung für eine vorteilhafte hohe Expression kann u.a. das Anhängen des spezifischen ER-Retentionssignals SEKDEL sein (Schouten, A. et al., Plant Mol. Biol. 30 (1996), 781-792), die durchschnittliche Expressionshöhe wird damit verdreifacht bis
5 vervierfacht. Es können auch andere Retentionssignale, die natürlicherweise bei im ER lokalisierten pflanzlichen und tierischen Proteinen vorkommen, für den Aufbau der Kassette eingesetzt werden.

- 10 Bevorzugte Polyadenylierungssignale sind pflanzliche Polyadenylierungssignale, vorzugsweise solche, die im wesentlichen T-DNA-Polyadenylierungssignale aus *Agrobacterium tumefaciens*, insbesondere des Gens 3 der T-DNA (Octopin Synthase) des Ti-Plasmids pTiACH5 entsprechen (Gielen et al., EMBO J.3 (1984),
15 835 ff) oder entsprechende funktionelle Äquivalente.

Die Herstellung einer Expressionskassette erfolgt durch Fusion eines geeigneten Promotors mit einer geeigneten $\Delta 6$ -Desaturase-DNA-Sequenz sowie einem Polyadenylierungssignal nach gängigen

- 20 Rekombinations- und Klonierungstechniken, wie sie beispielsweise in T. Maniatis, E.F. Fritsch und J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989) sowie in T.J. Silhavy, M.L. Berman und L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor
25 Laboratory, Cold Spring Harbor, NY (1984) und in Ausubel, F.M. et al., Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley-Interscience (1987) beschrieben werden.

Die DNA Sequenz codierend für eine $\Delta 6$ -Desaturase aus *Phsyco-*

- 30 *mitrella patens* beinhaltet alle Sequenzmerkmale, die notwendig sind, um eine dem Ort der Fettsäure-, Lipid- oder Ölbiosynthese korrekte Lokalisation zu erreichen. Daher sind keine weiteren Targetingsequenzen per se notwendig. Allerdings kann eine solche Lokalisation wünschenswert und vorteilhaft sein und daher künst-
35 lich verändert oder verstärkt werden, sodaß auch solche Fusionskonstrukte eine bevorzugte vorteilhafte Ausführungsform der Erfindung sind.

Insbesondere bevorzugt sind Sequenzen, die ein Targeting in

- 40 Plastiden gewährleisten. Unter bestimmten Umständen kann auch ein Targeting in andere Kompartimente (referiert: Kermode, Crit. Rev. Plant Sci. 15, 4 (1996), 285-423) z.B. in die Vakuole, in das Mitochondrium, in das Endoplasmatische Retikulum (ER), Peroxisomen, Lipidkörper oder durch ein Fehlen entsprechender
45 operativer Sequenzen ein Verbleib im Kompartiment des Entstehens, dem Zytosol, wünschenswert sein.

Vorteilhafterweise werden die für $\Delta 6$ -Desaturase-Gene codierenden Nukleinsäuresequenzen zusammen mit mindestens einem Reporter-gen in eine Expressionskassette kloniert, die in den Organismus über einen Vektor oder direkt in das Genom eingebracht wird. Dieses

- 5 Reporter-gen sollte eine leichte Detektierbarkeit über einen Wachstums-, Fluoreszenz-, Chemo-, Biolumineszenz- oder Resistenz-assay oder über eine photometrische Messung ermöglichen. Bei-spielhaft seien als Reporter-gene Antibiotika-oder Herbizid-resistenzgene, Hydrolasegene, Fluoreszenzproteingene, Biolumin-
10 eszenzgene, Zucker- oder Nukleotidstoffwechselgene oder Bio-synthesegene wie das Ura3-Gen, das Ilv2-Gen, das Luciferasegen, das β -Galactosidasegen, das gfp-Gen, das 2-Desoxyglucose-6-phosphat-Phosphatasegen, das β -Glucuronidase-Gen, β -Lactamasegen, das Neomycinphosphotransferasegen, das Hygromycinphosphotrans-
15 ferasegen oder das BASTA (= Gluphosinatresistenz)-Gen genannt. Diese Gene ermöglichen eine leichte Meßbarkeit und Quantifizier-barkeit der Transkriptionsaktivität und damit der Expression der Gene. Damit lassen sich Genomstellen identifizieren, die eine unterschiedliche Produktivität zeigen.

20

Gemäß einer bevorzugten Ausführungsform umfaßt eine Expressions-kassette stromaufwärts, d.h. am 5'-Ende der codierenden Sequenz, einen Promotor und stromabwärts, d.h. am 3'-Ende, ein Poly-adenylierungssignal und gegebenenfalls weitere regulatorische

- 25 Elemente, welche mit der dazwischenliegenden codierenden Sequenz für die $\Delta 6$ -Desaturase DNA Sequenz operativ verknüpft sind. Unter einer operativen Verknüpfung versteht man die sequenzielle Anordnung von Promotor, codierender Sequenz, Terminator und ggf. weiterer regulativer Elemente derart, daß jedes der regulativen
30 Elemente seine Funktion bei der Expression der codierenden Sequenz bestimmungsgemäß erfüllen kann. Die zur operativen Verknüpfung bevorzugten Sequenzen sind Targeting-Sequenzen zur Gewährleistung der subzellulären Lokalisation in Plastiden. Aber auch Targeting-Sequenzen zur Gewährleistung der subzellulären
35 Lokalisation im Mitochondrium, im Endoplasmatischen Retikulum (= ER), im Zellkern, in Ölkörperchen oder anderen Kompartimenten sind bei Bedarf einsetzbar sowie Translationsverstärker wie die 5'-Führungssequenz aus dem Tabak-Mosaik-Virus (Gallie et al., Nucl. Acids Res. 15 (1987), 8693-8711).

40

Eine Expressionskassette kann beispielsweise einen konstitutiven Promotor (bevorzugt den USP- oder Napin-Promotor), das zu exprimierende Gen und das ER-Retentionssignal enthalten. Als ER-Retentionssignal wird bevorzugt die Aminosäuresequenz KDEL

- 45 (Lysin, Asparaginsäure, Glutaminsäure, Leucin) verwendet.

- Die Expressionskassette wird zur Expression in einem prokaryontischen oder eukaryontischen Wirtsorganismus beispielsweise einem Mikroorganismus wie einem Pilz oder einer Pflanze vorteilhafterweise in einen Vektor wie beispielsweise einem Plasmid, einem Phagen oder sonstiger DNA inseriert, der eine optimale Expression der Gene im Wirtsorganismus ermöglicht. Geeignete Plasmide sind beispielsweise in *E. coli* pLG338, pACYC184, pBR-Serie wie z.B. pBR322, pUC-Serie wie pUC18 oder pUC19, M13mp-Serie, pKC30, pRep4, pHS1, pHS2, pPLc236, pMBL24, pLG200, pUR290, pIN-III¹¹³-B1, λ gt11 oder pBdCI, in *Streptomyces* pIJ101, pIJ364, pIJ702 oder pIJ361, in *Bacillus* pUB110, pC194 oder pBD214, in *Corynebacterium* pSA77 oder pAJ667, in Pilzen pALS1, pIL2 oder pBB116, weitere vorteilhafte Pilzvektoren werden von Romanos, M.A. et al., [(1992) "Foreign gene expression in yeast: a review", *Yeast* 8: 423-488] und von van den Hondel, C.A.M.J.J. et al. [(1991) "Heterologous gene expression in filamentous fungi" sowie in *More Gene Manipulations in Fungi* [J.W. Bennet & L.L. Lasure, eds., p. 396-428: Academic Press: San Diego] und in "Gene transfer systems and vector development for filamentous fungi" [van den Hondel, C.A.M.J.J. & Punt, P.J. (1991) in: *Applied Molecular Genetics of Fungi*, Peberdy, J.F. et al., eds., p. 1-28, Cambridge University Press: Cambridge] beschrieben. Vorteilhafte Hefektoren sind beispielsweise 2 μ M, pAG-1, YEp6, YEp13 oder pEMBLYe23. Beispiele für Algen- oder Pflanzenpromotoren sind pLGV23, pGHlac⁺, pBIN19, pAK2004, pVKH oder pDH51 (siehe Schmidt, R. and Willmitzer, L., 1988). Die oben genannten Vektoren oder Derivate der vorstehend genannten Vektoren stellen eine kleine Auswahl der möglichen Plasmide dar. Weitere Plasmide sind dem Fachmann wohl bekannt und können beispielsweise aus dem Buch *Cloning Vectors* (Eds. Pouwels P.H. et al. Elsevier, Amsterdam-New York-Oxford, 1985, ISBN 0 444 904018) entnommen werden. Geeignete pflanzliche Vektoren werden unter anderem in "Methods in Plant Molecular Biology and Biotechnology" (CRC Press), Kap. 6/7, S.71-119 beschrieben. Vorteilhafte Vektoren sind sog. shuttle-Vektoren oder binäre Vektoren, die in *E. coli* und *Agrobacterium* replizieren.

- Unter Vektoren sind außer Plasmiden auch alle anderen dem Fachmann bekannten Vektoren wie beispielsweise Phagen, Viren wie SV40, CMV, Baculovirus, Adenovirus, Transposons, IS-Elemente, Phasmide, Phagemide, Cosmide, lineare oder zirkuläre DNA zu verstehen. Diese Vektoren können autonom im Wirtsorganismus repliziert oder chromosomal repliziert werden, bevorzugt ist eine chromosomale Replikation.

In einer weiteren Ausgestaltungsform des Vektors kann die erfindungsgemäße Expressionskassette auch vorteilhafterweise in Form einer linearen DNA in die Organismen eingeführt werden und über heterologe oder homologe Rekombination in das Genom des

5 Wirtsorganismus integriert werden. Diese lineare DNA kann aus einem linearisierten Plasmid oder nur aus der Expressionskassette als Vektor oder den erfindungsgemäßen Nukleinsäuresequenzen bestehen.

10 In einer weiteren vorteilhaften Ausführungsform kann die erfindungsgemäße Nukleinsäuresequenz auch alleine in einen Organismus eingebracht werden.

Sollen neben der erfindungsgemäßen Nukleinsäuresequenz weitere

15 Gene in den Organismus eingeführt werden, so können alle zusammen mit einem Reportergen in einem einzigen Vektor oder jedes einzelne Gen mit einem Reportergen in je einem Vektor oder mehrere Gene zusammen in verschiedenen Vektoren in den Organismus eingebracht werden, wobei die verschiedenen Vektoren gleichzeitig

20 oder sukzessive eingebracht werden können.

Der Vektor enthält vorteilhaft mindestens eine Kopie der Nukleinsäuresequenzen, die für eine $\Delta 6$ -Desaturase codieren, und/oder der Expressionskassette.

25

Beispielhaft kann die pflanzliche Expressionskassette in den Transformationsvektor pRT ((a) Toepfer et al., 1993, Methods Enzymol., 217: 66-78; (b) Toepfer et al. 1987, Nucl. Acids. Res. 15: 5890 ff.) eingebaut werden.

30

Alternativ kann ein rekombinanter Vektor (= Expressionsvektor) auch in-vitro transkribiert und translatiert werden, z.B. durch Nutzung des T7 Promotors und der T7 RNA Polymerase.

35 In Prokaryoten verwendete Expressionsvektoren nutzen häufig induzierbare Systeme mit und ohne Fusionsproteinen bzw Fusions-oligopeptiden, wobei diese Fusionen sowohl N-terminal als auch C-terminal oder anderen nutzbaren Domänen eines Proteins erfolgen können. Solche Fusionsvektoren dienen in der Regel dazu: i.) die

40 Expressionsrate der RNA zu erhöhen ii.) die erzielbare Proteinsyntheserate zu erhöhen, iii.) die Löslichkeit des Proteins zu erhöhen, iv.) oder die Reinigung durch einen für die Affinitätschromatographie nutzbare Bindesequenz zu vereinfachen. Häufig werden auch proteolytische Spaltstellen über Fusionsproteine

45 eingeführt, was die Abspaltung eines Teils des Fusionsproteins auch der Reinigung ermöglicht. Solche Erkennungssequenzen für

Proteasen erkennen sind z.B. Faktor Xa, Thrombin und Entero-kinase.

Typische vorteilhafte Fusions- und Expressionsvektoren sind pGEX

- 5 [Pharmacia Biotech Inc; Smith, D.B. and Johnson, K.S. (1988) *Gene* 67: 31-40], pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) welches Glutathion S-transferase beinhaltet (GST), Maltose Bindeprotein, oder Protein A.

- 10 Weitere Beispiele für E. coli Expressionsvektoren sind pTrc [Amann et al., (1988) *Gene* 69:301-315] und pET Vektoren [Studier et al., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 60-89; *Stratagene*, Amsterdam, Niederlande].

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Weitere vorteilhafte Vektoren zur Verwendung in Hefe sind pYepSec1 (Baldari, et al., (1987) *Embo J.* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933-943), pJRY88 (Schultz et al., (1987) *Gene* 54:113-123), and pYES-Derivate (Invitrogen

- 20 Corporation, San Diego, CA). Vektoren für die Nutzung in filamentösen Pilzen sind beschrieben in: van den Hondel, C.A.M.J.J. & Punt, P.J. (1991) "Gene transfer systems and vector development for filamentous fungi, in: *Applied Molecular Genetics of Fungi*, J.F. Peberdy, et al., eds., p. 1-28, Cambridge University Press:

25 Cambridge.

Alternativ können auch vorteilhaft Insektenzellexpressionsvektoren genutzt werden z.B. für die Expression in Sf 9 Zellen. Dies sind z.B. die Vektoren der pAc Serie (Smith et al. (1983) *Mol.*

- 30 *Cell Biol.* 3:2156-2165) und der pVL series (Lucklow and Summers (1989) *Virology* 170:31-39).

Des weiteren können zur Genexpression vorteilhaft Pflanzenzellen oder Algenzellen genutzt werden. Beispiele für Pflanzen-

- 35 expressionsvektoren finden sich in Becker, D., et al. (1992)

"New plant binary vectors with selectable markers located proximal to the left border", *Plant Mol. Biol.* 20: 1195-1197 oder in Bevan, M.W. (1984) "Binary *Agrobacterium* vectors for plant transformation", *Nucl. Acid. Res.* 12: 8711-8721.

40

Weiterhin können die für die $\Delta 6$ -Desaturase codierenden Nukleinsäuresequenzen in Säugerzellen exprimiert werden. Beispiel für entsprechende Expressionsvektoren sind pCDM8 und pMT2PC genannt in: Seed, B. (1987) *Nature* 329:840 oder Kaufman et al.

- 45 (1987) *EMBO J.* 6: 187-195). Dabei sind vorzugsweise zu nutzende Promotoren viralen Ursprungs wie z.B. Promotoren des Polyoma, Adenovirus 2, Cytomegalovirus oder Simian Virus 40. Weitere

prokaryotische und eukaryotische Expressionssysteme sind genannt in Kapitel 16 und 17 in Sambrook et al., *Molecular Cloning: A Laboratory Manual*. 2nd, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 5 1989.

Das Einbringen der erfindungsgemäßen Nukleinsäuren, der Expressionskassette oder des Vektors in Organismen beispielsweise in Pflanzen kann prinzipiell nach allen dem Fachmann 10 bekannten Methoden erfolgen.

Für Mikroorganismen kann der Fachmann entsprechende Methoden den Lehrbüchern von Sambrook, J. et al. (1989) *Molecular cloning: A laboratory manual*, Cold Spring Harbor Laboratory Press, von 15 F.M. Ausubel et al. (1994) *Current protocols in molecular biology*, John Wiley and Sons, von D.M. Glover et al., *DNA Cloning Vol.1*, (1995), IRL Press (ISBN 019-963476-9), von Kaiser et al. (1994) *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory Press oder Guthrie et al. *Guide to Yeast Genetics and Molecular 20 Biology*, *Methods in Enzymology*, 1994, Academic Press entnehmen.

Die Übertragung von Fremdgenen in das Genom einer Pflanze wird als Transformation bezeichnet. Es werden dabei die beschriebenen Methoden zur Transformation und Regeneration von Pflanzen aus 25 Pflanzengeweben oder Pflanzenzellen zur transienten oder stabilen Transformation genutzt. Geeignete Methoden sind die Protoplastentransformation durch Polyethylenglykol-induzierte DNA-Aufnahme, das biolistische Verfahren mit der Genkanone - die sogenannte particle bombardment Methode -, die Elektroporation, die Inku- 30 bation trockener Embryonen in DNA-haltiger Lösung, die Mikroinjektion und der durch Agrobacterium vermittelte Gentransfer. Die genannten Verfahren sind beispielsweise in B. Jenes et al., *Techniques for Gene Transfer*, in: *Transgenic Plants*, Vol. 1, Engineering and Utilization, herausgegeben von S.D. Kung und R. 35 Wu, Academic Press (1993) 128-143 sowie in Potrykus *Annu. Rev. Plant Physiol. Plant Molec. Biol.* 42 (1991) 205-225) beschrieben. Vorzugsweise wird das zu exprimierende Konstrukt in einen Vektor kloniert, der geeignet ist, Agrobacterium tumefaciens zu transformieren, beispielsweise pBin19 (Bevan et al., *Nucl. Acids Res.* 40 12 (1984) 8711). Mit einem solchen Vektor transformierte Agrobakterien können dann in bekannter Weise zur Transformation von Pflanzen, insbesondere von Kulturpflanzen, wie z.B. von Tabakpflanzen, verwendet werden, indem beispielsweise verwundete Blätter oder Blattstücke in einer Agrobakterienlösung gebadet 45 und anschließend in geeigneten Medien kultiviert werden. Die Transformation von Pflanzen mit Agrobacterium tumefaciens wird beispielsweise von Höfgen und Willmitzer in *Nucl. Acid Res.*

(1988) 16, 9877 beschrieben oder ist unter anderem bekannt aus F.F. White, Vectors for Gene Transfer in Higher Plants; in Transgenic Plants, Vol. 1, Engineering and Utilization, herausgegeben von S.D. Kung und R. Wu, Academic Press, 1993, S. 15-38.

5

Mit einem wie oben beschriebenen Expressionsvektor transformierte Agrobakterien können ebenfalls in bekannter Weise zur Transformation von Pflanzen wie Testpflanzen wie Arabidopsis oder Kulturpflanzen wie Getreide, Mais, Hafer, Roggen, Gerste, Weizen,

- 10 Soja, Reis, Baumwolle, Zuckerrübe, Canola, Triticale, Reis, Sonnenblume, Flachs, Hanf, Kartoffel, Tabak, Tomate, Kaffee, Kakao, Tee, Karotte, Paprika, Raps, Tapioka, Maniok, Pfeilwurz, Tagetes, Alfalfa, Salat und den verschiedenen Baum-, Nuß- und Weinspezies, insbesondere von Öl-haltigen Kulturpflanzen,
- 15 wie Soja, Erdnuß, Rizinus, Borretsch, Lein, Sonnenblume, Canola, Baumwolle, Flachs, Raps, Kokosnuß, Ölpalme, Färbersaflor (*Carthamus tinctorius*) oder Kakaobohne verwendet werden, z.B. indem verwundete Blätter oder Blattstücke in einer Agrobakterienlösung gebadet und anschließend in geeigneten Medien kultiviert
- 20 werden.

Die genetisch veränderten Pflanzenzellen können über alle dem Fachmann bekannten Methoden regeneriert werden. Entsprechende Methoden können den oben genannten Schriften von S.D. Kung und

- 25 R. Wu, Potrykus oder Höfgen und Willmitzer entnommen werden.

Als Organismen bzw. Wirtsorganismen für die erfindungsgemäßen Verfahren verwendeten Nukleinsäuren, die verwendete Expressionskassette oder den verwendeten Vektor eignen sich prinzipiell

- 30 vorteilhaft alle Organismen, die in der Lage sind Fettsäuren speziell ungesättigte Fettsäuren zu synthetisieren bzw. für die Expression rekombinanter Gene geeignet sind. Beispielhaft seien Pflanzen wie Arabidopsis, Asteraceae wie Calendula oder Kulturpflanzen wie Soja, Erdnuß, Rizinus, Sonnenblume, Mais, Baum-
- 35 wolle, Flachs, Raps, Kokosnuß, Ölpalme, Färbersaflor (*Carthamus tinctorius*) oder Kakaobohne, Mikroorganismen wie Pilze beispielsweise die Gattung *Mortierella*, *Saprolegnia* oder *Pythium*, Bakterien wie die Gattung *Escherichia*, Cyanobakterien, Ciliaten, Thrausto- oder Schizichytrien, Algen oder Protozoen wie Dino-
- 40 flagellaten wie *Cryptocodium* genannt. Bevorzugt werden Organismen, die natürlicherweise Öle in größeren Mengen synthetisieren können wie Pilze der Gattungen *Mortierella* oder *Pythium* wie *Mortierella alpina*, *Pythium insidiosum* oder Pflanzen wie Soja, Raps, Kokosnuß, Ölpalme, Färbersaflor, Rizinus,
- 45 Calendula, Erdnuß, Kakaobohne oder Sonnenblume, besonders bevorzugt werden Soja, Raps, Sonnenblume, Rizinus, *Mortierella* oder

Pythium. Prinzipiell sind als Wirtsorganismen auch transgene Tiere geeignet beispielsweise *C. elegans*.

Nutzbare Wirtszellen sind weiterhin genannt in: Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990).

Verwendbare Expressionsstämme z.B. solche, die eine geringere Proteaseaktivität aufweisen sind beschrieben in: Gottesman, S., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 119-128.

Dabei kann je nach Wahl des Promotors die Expression des $\Delta 6$ -Desaturase-Gens spezifisch in den Blättern, in den Samen, den Knollen oder anderen Teilen der Pflanze erfolgen. Solche Fettsäuren, Öle oder Lipide mit $\Delta 6$ -Doppelbindungen überproduzierenden transgenen Pflanzen, deren Vermehrungsgut, sowie deren Pflanzenzellen, -gewebe oder -teile, sind ein weiterer Gegenstand der vorliegenden Erfindung. Ein bevorzugter erfindungsgemäßer Gegenstand sind transgene Pflanzen beispielsweise Kulturpflanzen wie Mais, Hafer, Roggen, Weizen, Gerste, Reis, Soja, Zuckerrübe, Canola, Triticale, Sonnenblume, Flachs, Hanf, Tabak, Tomate, Kaffee, Kakao, Tee, Karotte, Paprika, Raps, Tapioka, Maniok, Pfeilwurz, Tagetes, Alfalfa, Salat und den verschiedenen Baum-, Nuß- und Weinspezies, Kartoffel, insbesondere Öl-haltige Kulturpflanzen, wie Soja, Erdnuß, Rizinus, Borretsch, Lein, Sonnenblume, Canola, Baumwolle, Flachs, Raps, Kokosnuß, Ölpalme, Färbersaflor (*Carthamus tinctorius*) oder Kakaobohne, Testpflanzen wie *Arabidopsis* oder sonstige Pflanzen wie Moose oder Algen enthaltend eine erfindungsgemäße funktionelle Nukleinsäuresequenz oder eine funktionelle Expressionskassette. Funktionell bedeutet hierbei, daß ein enzymatisch aktives Enzym gebildet wird.

Die Expressionskassette oder die erfindungsgemäßen Nukleinsäuresequenzen enthaltend eine $\Delta 6$ -Desaturasegenesequenz kann darüber hinaus auch zur Transformation der oben beispielhaft genannten Organismen wie Bakterien, Cyanobakterien, filamentösen Pilzen, Ciliaten, Tiere oder Algen mit dem Ziel einer Erhöhung des Gehaltes an Fettsäuren, Ölen oder Lipiden $\Delta 6$ -Doppelbindungen eingesetzt werden. Bevorzugte transgene Organismen sind Bakterien, Cyanobakterien, filamentöse Pilze oder Algen.

Unter transgenen Organismen sind Organismen zu verstehen, die eine Fremde aus einem anderen Organismus stammende Nukleinsäure, die für eine im erfindungsgemäßen Verfahren verwendete $\Delta 6$ -Desaturase codiert, enthalten. Unter transgenen Organismen sind auch Organismen zu verstehen, die eine Nukleinsäure, die

aus demselben Organismus stammt und die für eine $\Delta 6$ -Desaturase codiert, enthält, wobei diese Nukleinsäure als zusätzliche Genkopie enthalten ist oder nicht in der natürlichen Nukleinsäureumgebung des $\Delta 6$ -Desaturase-Gens enthalten ist. Transgene

5 Organismen sind auch Organismen bei denen die natürliche 3'- und/oder 5'-Region des $\Delta 6$ -Desaturase-Gens durch gezielte gentechnologische Veränderungen gegenüber dem Ausgangsorganismus verändert wurde. Bevorzugt sind transgene Organismen bei denen eine Fremd-DNA eingebracht wurde. Besonders bevorzugt sind trans-
10 gene Pflanzen, in die Fremd-DNA eingebracht wurde. Unter transgenen Pflanzen sind einzelne Pflanzenzellen und deren Kulturen wie beispielsweise Kalluskulturen auf Festmedien oder in Flüssigkultur, Pflanzenteile und ganze Pflanzen zu verstehen.

15 Ein weiterer Erfindungsgegenstand sind transgene Organismen ausgewählt aus der Gruppe Pflanzen, Pilze, Ciliaten, Algen, Bakterien, Cyanobakterien oder Tiere, bevorzugt transgene Pflanzen oder Algen, die mindestens eine isolierte Nukleinsäuresequenz enthalten, die für ein Polypeptid mit $\Delta 6$ -Desaturase-
20 aktivität codiert, ausgewählt aus der Gruppe:

a) einer Nukleinsäuresequenz mit der in SEQ ID NO: 1 dargestellten Sequenz,

25 b) Nukleinsäuresequenzen, die sich als Ergebnis des degenerierten genetischen Codes von der in SEQ ID NO: 1 ableiten

c) Derivate der in SEQ ID NO: 1 dargestellten Nukleinsäuresequenz, die für Polypeptide mit der in SEQ ID NO: 2
30 dargestellten Aminosäuresequenzen codieren und mindestens 50 % Homologie auf Aminosäureebene aufweisen, ohne daß die enzymatische Wirkung der Polypeptide wesentlich reduziert ist.

35 Erhöhung des Gehaltes von Fettsäuren, Ölen oder Lipiden mit $\Delta 6$ -Doppelbindungen bedeutet im Rahmen der vorliegenden Erfindung beispielsweise die künstlich erworbene Fähigkeit einer erhöhten Biosyntheseleistung durch funktionelle Überexpression des $\Delta 6$ -Desaturase-Gens in den erfindungsgemäßen Organismen vorteil-
40 haft in den erfindungsgemäßen transgenen Pflanzen gegenüber den nicht gentechnisch modifizierten Ausgangspflanzen zumindest für die Dauer mindestens einer Pflanzengeneration.

Der Biosyntheseort von Fettsäuren, Ölen oder Lipiden beispielsweise
45 weise ist im allgemeinen der Samen oder Zellschichten des Samens, so daß eine samenspezifische Expression des $\Delta 6$ -Desaturase-Gens sinnvoll ist. Es ist jedoch naheliegend, daß die Biosynthese

von Fettsäuren, Ölen oder Lipiden nicht auf das Samengewebe beschränkt sein muß, sondern auch in allen übrigen Teilen der Pflanze - beispielsweise in Epidermiszellen oder in den Knollen-gewebe spezifisch erfolgen kann.

5

Darüberhinaus ist eine konstitutive Expression des exogenen $\Delta 6$ -Desaturase-Gens von Vorteil. Andererseits kann aber auch eine induzierbare Expression wünschenswert erscheinen.

- 10 Die Wirksamkeit der Expression des $\Delta 6$ -Desaturase-Gens kann beispielsweise *in vitro* durch Sproßmeristemvermehrung ermittelt werden. Zudem kann eine in Art und Höhe veränderte Expression des $\Delta 6$ -Desaturase-Gens und deren Auswirkung auf die Fettsäure-, Öl- oder Lipidbiosyntheseleistung an Testpflanzen in Gewächshaus-
15 versuchen getestet werden.

Gegenstand der Erfindung sind wie oben beschrieben transgene Pflanzen, transformiert mit einer Nukleinsäuresequenz, die für eine $\Delta 6$ -Desaturase codiert, einem Vektor oder einer Expressions-

- 20 kassette enthaltend eine $\Delta 6$ -Desaturase-Gensequenz oder mit dieser hybridisierende DNA-Sequenzen, sowie transgene Zellen, Gewebe, Teile und Vermehrungsgut solcher Pflanzen. Besonders bevorzugt sind dabei transgene Kulturpflanzen wie oben beschrieben.

- 25 Pflanzen im Sinne der Erfindung sind mono- und dikotyle Pflanzen oder Algen.

Weitere Gegenstände der Erfindung sind:

- 30 - Verwendung einer $\Delta 6$ -Desaturase-DNA-Gensequenz mit der in SEQ ID NO:1 genannten Sequenz oder mit dieser hybridisierende DNA-Sequenzen zur Herstellung von Pilzen, Bakterien, Tieren oder Pflanzen bevorzugt Pflanzen mit erhöhtem Gehalt an Fettsäuren, Ölen oder Lipiden mit $\Delta 6$ -Doppelbindungen durch
35 Expression dieser $\Delta 6$ -Desaturase DNA-Sequenz in Pflanzen.
- Verwendung der Proteine mit den Sequenzen SEQ ID NO: 2 zur Herstellung von ungesättigten Fettsäuren in Pflanzen, Pilzen, Bakterien oder Tieren bevorzugt Pflanzen.

40

45

Die Erfindung wird durch die folgenden Beispiele näher erläutert:

Beispiele

5 Beispiel 1: Allgemeine Klonierungsverfahren und Anzuchtungsverfahren:

- Die Klonierungsverfahren wie z.B. Restriktionsspaltungen, Agarose-Gelelektrophorese, Reinigung von DNA-Fragmenten, Transfer
10 von Nukleinsäuren auf Nitrozellulose und Nylon Membranen, Verknüpfen von DNA-Fragmenten, Transformation von *Escherichia coli* Zellen, Anzucht von Organismen und die Sequenzanalyse rekombinanter DNA wurden wie bei Sambrook et al. (1989) (Cold Spring Harbor Laboratory Press: ISBN 0-87969-309-6) beschrieben durchgeführt.
15 Das Protonema von *Physcomitrella patens* (= *P. patens*) wurde in Flüssigmedium, wie von Reski et al. (Mol. Gen. Genet., 244, 1994: 352-359) beschrieben, angezogen.

Beispiel 2: Sequenzanalyse rekombinanter DNA

- 20 Die Sequenzierung rekombinanter DNA-Moleküle erfolgte mit einem Laserfluoreszenz-DNA-Sequenzierer der Firma ABI nach der Methode von Sanger (Sanger et al. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467). Fragmente resultierend aus einer Polymerase Ketten-
25 reaktion wurden zur Vermeidung von Polymerasefehlern in zu exprimierenden Konstrukten sequenziert und überprüft.

Beispiel 3: Lipidanalyse aus dem Protonema von *P. patens* und aus Hefezellen

- 30 Die Lipide wurden mit Chloroform/Methanol wie bei Siebertz et al. (Eur. J. Biochem., 101, 1979: 429-438) beschrieben aus dem Protonema von *S. patens* oder aus Hefezellen extrahiert und über Dünnschichtchromatographie (= TLC) mit Diethylether ge-
35 reinigt. Die erhaltenen Fettsäuren wurden zu den entsprechenden Methylestern transmethyliert und mit Gaschromatographie (= GC) analysiert. Die verschiedenen Methylester wurden mit den entsprechenden Standards identifiziert. Entsprechende Fettsäurepyrrolididen wurden, wie bei Anderson et al. (Lipids, 9, 1974:
40 185-190) beschrieben, erhalten und mit GC-MS bestimmt.

27

Beispiel 4: Funktionelle Expression der $\Delta 6$ -Desaturase cDNA von *P. patens* in Hefen

Die Expression-Experimente in Hefen wurden mit PPDES6-cDNA durchgeführt. Knock-out-Experimente hatten gezeigt (Daten und Versuchsdurchführung nicht gezeigt bzw. beschrieben), daß der Knock-out zu einem Verlust an 20:3^{11,14,17}-, 20:4^{5,8,11,14}-, 20:4^{5,11,14,17}- und 20:5^{5,8,11,14,17}-Fettsäuren führt. Gleichzeitig steigen die 18:2^{9,12}- und 18:3^{9,12,15}-Fettsäuren an. Für die Expression in Hefe wurde der PPDES6-cDNA in den Hefe-Expressionsvektor pYES2 (Invitrogen) subkloniert. Der erhaltene Vektor erhielt die Bezeichnung pYESdelta6. Mit pYES2 (Kontrolle) und pYESdelta6 ($\Delta 6$ -Desaturase-cDNA) transformierte Hefekulturen wurden auf Uracil-dop-out Medium mit 2 % Raffinose und 1 % Tergitol NP-40 (zur Stabilisierung der Fettsäuren) angezogen. Für die Expression wurden die Zellen mit Galactose (Endkonzentration 2 %) bis zu einer optischen Dichte (= OD) von 0,5 bei 600nm angezogen. In Fütterungsexperimenten wurden Fettsäuren in 5 % Tergitol solubilisiert und mit einer Endkonzentration von 0,0003 % zugesetzt. Die Ergebnisse der Expression sind Tabelle I zu entnehmen. Die Synthese von Fettsäuren mit einer Doppelbindung an Position 6 ist nur in Gegenwart des Expressionskonstrukts mit der $\Delta 6$ -Desaturase-cDNA möglich. Dieses $\Delta 6$ -Desaturase-Enzym hat eine größere Aktivität gegenüber Fettsäuren, die schon eine Doppelbindung an Position 9 oder 12 (Bezug auf Kohlenstoffatom in der Kette) enthalten. Es wurden die Fettsäuremethylester des gesamten Lipids der Hefen mit GC analysiert. Die einzelnen synthetisierten Fettsäuren werden in der Tabelle in Mol-% der gesamten Fettsäuren angegeben.

Tabelle I: Fettsäurezusammensetzung in transformierten Hefen gegenüber der Kontrolle

Gesamt Fettsäure (%)				
	pYES2		pYESdelta6	
Fettsäuren	-	-	+ 18:2 ^{9,12}	+18:3 ^{9,12,15}
16:0	16,4	16,1	23,8	25,8
16:1 ⁹	54,0	55,5	38,1	31,4
16:2 ^{6,9}	-	4,2	1,7	-
18:0	3,2	2,4	4,0	-
18:1 ⁹	24,9	19,7	19,1	19,2
18:2 ^{6,9}	-	0,6	0,2	-
18:2 ^{9,12}	-	-	8,5	-
18:3 ^{6,9,12}	-	-	4,0	-
18:3 ^{9,12,15}	-	-	-	11,7
18:4 ^{6,9,12,15}	-	-	-	3,0

Beispiel 5: Transformation von *P. patens*

- Die Polyethylenglycol vermittelte direkte DNA-Transformation von Protoplasten wurde, wie von Schäfer et al. (Mol. Gen. Genet., 5 226, 1991: 418-424) beschrieben, durchgeführt. Die Selektion der Transformanten erfolgte auf G418-enthaltenden Medium (Girke et al., The Plant Journal, 15, 1998: 39-48).

10 Beispiel 6: Isolierung von $\Delta 6$ -Desaturase cDNA und genomischen Clonen von *P. patens*

Mit Hilfe eines PCR-Ansatzes mit den folgenden degenerierten Oligonukleotiden als Primer:

- 15 A: TGGTGGAA(A/G)TGGA(C/A)ICA(T/C)AA und
B: GG(A/G)AA(A/C/G/T)A(A/G)(G/A)TG(G/A)TG(C/T)TC]

und dem folgenden Temperaturprogramm:

- 94°C, 3 min; [94°C, 20 sec; 45°C, 30 sec; 72°C, 1 min], 30 Zyklen;
20 72°C, 5 min, wurden schließlich Fragmente einer $\Delta 6$ -Desaturase-Gen kloniert. Für die Klonierung wurde poly(A)RNA aus 12 Tage alten *P. patens* Protonema-Kultur isoliert. Mit dieser poly(A)RNA wurde die oben beschriebene PCR durchgeführt. Fragmente der erwarteten Fragmentlänge (500 bis 600 bp) wurden in pUC18 kloniert und
25 sequenziert. Die abgeleitete Aminosäuresequenz eines PCR-Fragments zeigte Ähnlichkeiten zu bekannten $\Delta 6$ -Desaturasen. Da bekannt war, daß *P. patens* eine $\Delta 6$ -Desaturase besitzt, wurde angenommen, daß dieser Klon für einen Teil einer $\Delta 6$ -Desaturase codiert.
- 30 Ein vollständiger cDNA-Klon (= PPDES6-cDNA) wurde aus einer *P. patens* cDNA-Bank von 12 Tage alten Protonemata mit Hilfe des oben genannten PCR-Fragments isoliert. Die Nukleotidsequenz wird in SEQ ID NO:1 wiedergegeben. Die abgeleitete Aminosäuresequenz ist SEQ ID NO:2 zu entnehmen. Die zugehörige genomische Sequenz
35 (= PPDES6-Gen) konnte mit Hilfe der PCR und den folgenden Oligonukleotiden als Primer isoliert werden:

C: CCGAGTCGCGGATCAGCC

D: CAGTACATTTCGGTCATTACAC:

40

Tabelle II gibt die Ergebnisse des Vergleichs zwischen der neuen *P. patens* $\Delta 6$ -Desaturase über die gesamte Nukleinsäuresequenz mit folgenden bekannten $\Delta 6$ -Desaturase wieder: *Borago officinalis* (U79010), *Synechocystis* sp (L11421), *Spirulina platensis* (X87094), *Caenorhabditis elegans* (AF031477), *Mortierella alpina* (WO 98/46764), *Homo sapiens* (Cho et al., J. Biol. Chem., 274, 1999: 471-477), *Rattus norvegicus* (AB021980) und *Mus musculus*

29

(Cho et al., J. Biol. Chem., 274, 1999: 471-477). Die Analyse wurde mit dem Gap Programm (GCG-Package, Version 9,1) und den folgenden Analysenparametern durchgeführt: scoring matrix, blosum62, gap creation penalty, 12; gap extension penalty, 4.

- 5 Die Ergebnisse geben die bestimmte Identität oder Ähnlichkeit [] in Prozent (%) im Vergleich zur *P. patens*-Sequenz wieder.

Tabelle II: Sequenzvergleich zwischen *P. patens* $\Delta 6$ -Desaturase und anderen $\Delta 6$ -Desaturasen

10

Sequenz	Aminosäuresequenz-Identität [Ähnlichkeit] (%)
Borago officinalis	31 [38]
Synechocystis sp.	21 [29]
15 Spirulina platensis	20 [29]
Caenorhabditis elegans	35 [43]
Mortierella alpina	39 [47]
Homo sapiens	27 [38]
20 Rattus norvegicus	28 [39]
Mus musculus	29 [39]

Beispiel 7: Klonierung der $\Delta 6$ -Desaturase aus *Physcomitrella patens*

25

Die genomische $\Delta 6$ -Acyl lipid-Desaturase aus *Physcomitrella patens* wurde auf Grundlage der veröffentlichten Sequenz (Girke et al., Plant J., 15, 1998: 39-48) mittels Polymerasekettenreaktion und

30 Klonierung modifiziert, isoliert und für das erfindungsgemäße Verfahren eingesetzt. Dazu wurde zunächst mittels Polymerasekettenreaktion unter Verwendung von zwei genspezifischen Primern ein Desaturase-Fragment isoliert und in das bei Girke et al. (siehe oben) beschriebene Desaturasegen eingesetzt.

35

Primer TG5: 5'- ccgctcgagcgagggttggttgaggagcggc und
Primer TG3: 5'-ctgaaatagctcttgctcc-3'

dienten zunächst zur Amplifizierung eines Genfragmentes mittels

40 Polymerasekettenreaktion (30 Zyklen, 30 sek. 94° V, 30 sek. 50°C, 60 sek. 72°C, 10 min Nachinkubation bei 72°C, in einem Perkin Elmer Thermocycler).

45

- a) Klonierung eines Expressionsplasmids, das die $\Delta 6$ -Desaturase unter Kontrolle des 35S CaMV Promotors exprimiert:

5 Durch Primer TG5 wurde eine XhoI Schnittstelle in das
Fragment eingeführt. Ein XhoI/Eco47III Fragment wurde durch
Restriktion erhalten und in die bei Girke et al. beschriebene
PPDES6-Gensequenz nach analoger Restriktion mit XhoI/Eco47III
ausgetauscht. Das Konstrukt erhielt den Namen pZK. Das Insert
10 von pZK wurde als XhoI/HindIII Fragment nach Auffüllen der
HindIII-Schnittstelle mit Nukleotiden durch Behandlung mit
dem Klenow Fragment der DNA Polymerase I in die XhoI/SmaI
Schnittstellen von pRT99/35S kloniert. Das resultierende
Plasmid pSK enthält den 35S-Promotor [Cauliflower-Mosaik-Vi-
15 rus, Franck et al. (1980) Cell 21, 285], die $\Delta 6$ -Desaturase
aus Moos und den 35S-Terminator im Vektor pRT.

- b) Konstruktion eines Expressionskonstruktes unter Kontrolle
des Napin-Promotors:

20 Durch Schneiden des Plasmides pSK mit XhoI, Behandlung mit
T4-DNA Polymerase und PstI-Restriktion wurde das erhaltene
Promotor-Desaturase-Fragment mit Terminator in den Vektor
pJH3 kloniert. Dazu wurde der Vektor BamHI geschnitten und
mit Klenow-Enzym die Überhänge aufgefüllt sowie anschließend
25 mit PstI nachgeschnitten. Es entstand durch Ligation des
Desaturase-Terminator-Fragmentes in den Vektor das Plasmid
pJH7, das einen Napin-Promotor beinhaltet (Scofield et al.,
1987, J. Biol. Chem. 262, 12202-8). Die Expressionskassette
aus pJH7 wurde mit Bsp120I und NotI geschnitten und in den
30 binären Vektor pRE kloniert. Es entstand das Plasmid pRE-
Ppdes6.

In einer PCR Reaktion wurde die erfindungsgemäße
35 $\Delta 6$ -Desaturase cDNA aus *P. patens* als Matrize verwendet.
Mithilfe der nachfolgend aufgeführten Oligonukleotide wurde
eine BamHI-Restriktionsschnittstelle vor dem Startcodon und
drei Adeninnukleotide als Konsensustranslationssequenz für
Eukaryoten in die $\Delta 6$ -Desaturase cDNA eingeführt. Es wurde
ein 1512 Basenpaarfragment der $\Delta 6$ -Desaturase amplifiziert
40 und sequenziert.

Pp-d6Des1: 5'- CC GGTACC aaaatggtattcgcgggcggtg -3'
Pp-d6Des2: 3'- CC GGTACC ttaactggtggttagcatgct -3'

45 Die Reaktionsgemische enthielten ca. 1 ng/micro l Matrizen
DNA, 0,5 μ M der Oligonukleotide und, 200 μ M Desoxy-Nukleotide
(Pharmacia), 50 mM KCl, 10 mM Tris-HCl (pH 8,3 bei 25°C,

31

1,5 mM MgCl₂) und 0,02 U/µl Pwo Polymerase (Boehringer Mannheim) und werden in einer PCR-Maschine der Firma Perkin Elmer mit folgendem Temperaturprogramm inkubiert:

- 5 Anlagerungstemperatur: 50°C, 30 sec
 Denaturierungstemperatur: 95°C, 30 sec
 Elongationstemperatur: 72°C, 90 sec
 Anzahl der Zyklen: 30

- 10 c) Konstruktion eines Expressionskonstruktes unter Kontrolle des USP-Promotors:
Das erhaltene Fragment von ca. 1,5 kB Basenpaaren wurde in den mit EcoRV gespaltenen Vektor pBluescript SK- (Stratagene) ligiert und stand für weitere Klonierungen als BamHI Fragment
15 zur Verfügung.

- Für die Transformation von Pflanzen wurde ein weiterer Transformationsvektor auf Basis von pBin-USP erzeugt, der das BamHI-Fragment der Δ6-Desaturase enthält. pBin-USP ist ein
20 Derivat des Plasmides pBin19. pBinUSP entstand aus pBin19, indem in pBin19 [Bevan et al. (1980) Nucl. Acids Res. 12, 8711] ein USP-Promotor als EcoRI-BamHI-Fragment inseriert wurde. Das Polyadenylierungssignal ist das des Gens 3 der T-DNA des Ti-Plasmides pTiACH5 (Gielen et al., (1984) EMBO
25 J. 3, 835), wobei Nukleotide 11749-11939 als PvuII-HindIII-Fragment isoliert und nach Addition von SphI-Linkern an die PvuII-Schnittstelle zwischen die SphI-HindIII Schnittstelle des Vektors kloniert. Der USP-Promotor entspricht den Nukleotiden 1-684 (Genbank Accession X56240), wobei ein Teil der
30 nichtcodierenden Region des USP-Gens im Promotor enthalten ist. Das 684 Basenpaar große Promotorfragment wurde mittels käuflichen T7-Standardprimer (Stratagene) und mit Hilfe eines synthetisierten Primers über eine PCR-Reaktion nach Standardmethoden amplifiziert (Primersequenz: 5'-GTCGACCCGCGGACTAGTGGCCCTCTAGACCCGGGGGATCC GGATCTGCTGGCTATGAA-3'). Das PCR-
35 Fragment wurde mit EcoRI/SalI nachgeschnitten und in den Vektor pBin19 mit OCS Terminator eingesetzt. Es entstand das Plasmid mit der Bezeichnung pBinUSP.

- 40 d) Konstruktion eines Expressionskonstruktes unter Kontrolle des vATPase-C1-Promotors aus Beta vulgaris:

- Analog zum Expressionsplasmid mit dem USP-Promotor wurde ein Konstrukt unter Verwendung des v-ATPase-cl-Promotors
45 erstellt. Der Promotor wurde als EcoRI/KpnI Fragment in das Plasmid pBin19 mit OCS Terminator kloniert und über BamHI das Δ6-Desaturasegen aus P. patens zwischen Promotor und

32

Terminator inseriert. Der Promotor entspricht einem 1153 Basenpaarfragment aus beta-Vulgaris (Plant Mol Biol, 1999, 39:463-475).

- 5 Das Konstrukt wurde zur Transformation von Arabidopsis thaliana und Rapspflanzen eingesetzt.

Beispiel 8: Erzeugung transgener Rapspflanzen (verändert nach Moloney et al., 1992, Plant Cell Reports, 8:238-242)

10

Zur Erzeugung transgener Rapspflanzen wurden binäre Vektoren in Agrobacterium tumefaciens C58C1:pgV2260 oder Escherichia coli genutzt (Deblaere et al, 1984, Nucl. Acids. Res. 13, 4777-4788). Zur Transformation von Rapspflanzen (Var. Drakkar, NPZ Nord-

- 15 deutsche Pflanzenzucht, Hohenlieth, Deutschland), wurde eine 1:50 Verdünnung einer Übernachtskultur einer positiv transformierten Agrobakterienkolonie in Murashige-Skoog Medium (Murashige und Skoog 1962 Physiol. Plant. 15, 473) mit 3 % Saccharose (3MS-Medium) benutzt. Petiolen oder Hypokotyledonen frisch gekeimter
20 steriler Rapspflanzen (zu je ca. 1 cm²) wurden in einer Petrischale mit einer 1:50 Agrobakterienverdünnung für 5-10 Minuten inkubiert. Es folgte eine 3-tägige Inkubation in Dunkelheit bei 25°C auf 3MS-Medium mit 0,8 % Bacto-Agar. Die Kultivierung wurde nach 3 Tagen mit 16 Stunden Licht/8 Stunden Dunkelheit weiter-
25 geführt und in wöchentlichem Rhythmus auf MS-Medium mit 500 mg/l Claforan (Cefotaxime-Natrium), 50 mg/l Kanamycin, 20 µM Benzylaminopurin (BAP) und 1,6 g/l Glukose weitergeführt. Wachsende Sprosse wurden auf MS-Medium mit 2 % Saccharose, 250 mg/l Claforan und 0,8 % Bacto-Agar überführt. Bildeten sich nach
30 drei Wochen keine Wurzeln, so wurde als Wachstumshormon 2-Indolbuttersäure zum Bewurzeln zum Medium zugegeben.

Regenerierte Sprosse wurden auf 2MS-Medium mit Kanamycin und Claforan erhalten, nach Bewurzelung in Erde überführt und

- 35 nach Kultivierung für zwei Wochen in einer Klimakammer oder im Gewächshaus angezogen, zur Blüte gebracht, reife Samen geerntet und auf $\Delta 6$ -Desaturase-Expression mittels Lipidanalysen untersucht. Linien mit erhöhten Gehalten an oder Doppelbindungen an der $\Delta 6$ -Position wurden identifiziert. Es konnte in den stabil
40 transformierten transgenen Linien, die das Transgen funktionell exprimierten, ein erhöhter Gehalt von Doppelbindungen an der $\Delta 6$ -Position im Vergleich zu untransformierten Kontrollpflanzen festgestellt werden.

45

Beispiel 8: Lipidextraktion aus Samen

Das Pflanzenmaterial wurde zunächst mechanisch durch Mörsern homogenisiert, um es einer Extraktion zugänglicher zu machen.

5 Dann wurde es 10 min bei 100°C abgekocht und nach dem Abkühlen auf Eis sedimentiert. Das Zellsediment wurde mit 1 N methanolischer Schwefelsäure und 2 % Dimethoxypropan 1h bei 90°C hydrolysiert und die Lipide transmethyliert. Die resultierenden Fettsäure-
10 methylester (FAME) wurden in Petrolether extrahiert. Die extrahierten FAME wurden durch Gasflüssigkeitschromatographie mit einer Kapillarsäule (Chrompack, WCOT Fused Silica, CP-Wax-52 CB, 25 m, 0,32 mm) und einem Temperaturgradienten von 170°C auf 240°C in 20 min und 5 min bei 240°C analysiert. Die Identität der Fett-
15 säuremethylester wurde durch Vergleich mit entsprechenden FAME-Standards (Sigma) bestätigt. Die Identität und die Position der Doppelbindung konnte durch geeignete chemische Derivatisierung der FAME-Gemische z.B. zu 4,4-Dimethoxyoxazolin-Derivaten (Christie, 1997, in: Advances in Lipid Methodology, 4. Auflage:
20 Christie, Oily Press, Dundee, 119-169, und 1998, Gaschromatographie-Massenspektrometrie Verfahren, Lipide 33:343-353) mittels GC-MS weiter analysiert werden. Die GC-Analysen der Fettsäuremethylester aus den transgenen Rapssamen, die samenspezifisch die $\Delta 6$ -Desaturase exprimierten sind in Tabelle III dargestellt. Die
25 transgenen Rapssamen weisen mindestens 4,95 % γ -Linolensäure im Samen auf.

Tabelle III gibt die GC-Analysen der Fettsäuremethylester aus reifen, transgenen Rapssamen, die $\Delta 6$ -Desaturase samen-
30 spezifisch exprimieren, wieder. Die Fettsäurezusammensetzung ist in [mol %] der Gesamtfettsäuren angegeben. Es ist festzustellen, daß einzelne Pflanzen der T2 Generation, die aus positiv transformierten und geselbsteten Pflanzen erhalten wurden, bis zu ca. 4,95 % γ -Linolensäure enthalten.

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Tabelle III: GC-Analysen der Fettsäuremethylester von Raps

	Bezeichnung	18:0	18:1	18:2	18:3 (γ)	18:3 (α)	18:4
5	R2-T2-11/1a	1,98	53,58	22,63	3,86	11,38	0
	R2-T2-11/1b	1,86	52,04	25,45	2,31	11,39	0
	R2-T2-11/1c	1,95	49,17	24,30	2,84	9,20	0
	R2-T2-11/3	1,82	49,83	24,54	3,88	10,12	0
	R2-T2-11/4	1,72	48,02	24,66	4,95	9,52	0
	R2-T2-11/5a	1,73	51,98	25,27	4,27	9,61	0
10	R2-T2-11/5b	2,02	56,19	25,08	0	9,33	0
	R2-T2-11/5c	2,01	46,95	27,38	0	10,37	0
	R2-T2-11/5d	1,83	49,49	24,15	4,40	8,65	0
	R2-T2-11/6	2,08	54,52	23,94	2,05	9,29	0
	R2-T2-11/10	1,94	53,92	22,81	4,06	9,44	0
15	R2-T2-WT	1,90	47,75	30,91	0	10,51	0

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Patentansprüche

1. Verfahren zur Herstellung von ungesättigten Fettsäuren, da-
5 durch gekennzeichnet, daß mindestens eine isolierte Nuklein-
säuresequenz, die für ein Polypeptid mit $\Delta 6$ -Desaturase-
aktivität codiert, ausgewählt aus der Gruppe:
 - a) einer Nukleinsäuresequenz mit der in SEQ ID NO: 1 dar-
10 gestellten Sequenz,
 - b) Nukleinsäuresequenzen, die sich als Ergebnis des
degenerierten genetischen Codes von der in SEQ ID NO: 1
15 ableiten
 - c) Derivate der in SEQ ID NO: 1 dargestellten Nukleinsäure-
sequenz, die für Polypeptide mit der in SEQ ID NO: 2 dar-
20 gestellten Aminosäuresequenzen codieren und mindestens
50 % Homologie auf Aminosäureebene aufweisen, ohne daß
die enzymatische Wirkung der Polypeptide wesentlich
reduziert ist,
- in einen Organismus eingebracht wird, dieser Organismus
angezogen wird, wobei der angezogene Organismus mindestens
25 1 Mol-% ungesättigte Fettsäuren bezogen auf den gesamten
Fettsäuregehalt im Organismus enthält.
2. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß die
Nukleinsäuresequenz von einer Pflanze oder Alge stammt.
30
3. Verfahren nach Anspruch 1 oder 2, dadurch gekennzeichnet,
daß die Nukleinsäuresequenz von *Physcomitrella patens* stammt.
4. Verfahren nach den Ansprüchen 1 bis 3, dadurch gekenn-
35 zeichnet, daß es sich bei dem Organismus um ein organismus
ausgewählt aus der Gruppe Bakterium, Pilz, Ciliat, Alge,
Cyanobakterium, Tier oder Pflanze handelt.
5. Verfahren nach den Ansprüchen 1 bis 4, dadurch gekenn-
40 zeichnet, daß es sich bei dem Organismus um eine Pflanze
oder Alge handelt.

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6. Verfahren nach den Ansprüchen 1 bis 5, dadurch gekennzeichnet, daß es sich bei dem Organismus um eine Ölfruchtpflanzen handelt.
- 5 7. Verfahren nach den Ansprüchen 1 bis 6, dadurch gekennzeichnet, daß der angezogene Organismus mindestens 5 Gew-% ungesättigte Fettsäuren bezogen auf den gesamten Fettsäuregehalt im Organismus enthält.
- 10 8. Verfahren nach den Ansprüchen 1 bis 7, dadurch gekennzeichnet, daß die ungesättigten Fettsäuren aus dem Organismus isoliert werden.
9. Transgener Organismus ausgewählt aus der Gruppe Pflanzen,
15 Pilze, Ciliaten, Algen, Bakterien, Cyanobakterien oder Tiere, die mindestens eine isolierte Nukleinsäuresequenz enthalten, die für ein Polypeptid mit $\Delta 6$ -Desaturaseaktivität codiert, ausgewählt aus der Gruppe:
- 20 a) einer Nukleinsäuresequenz mit der in SEQ ID NO: 1 dargestellten Sequenz,
- b) Nukleinsäuresequenzen, die sich als Ergebnis des degenerierten genetischen Codes von der in SEQ ID NO: 1
25 ableiten
- c) Derivate der in SEQ ID NO: 1 dargestellten Nukleinsäuresequenz, die für Polypeptide mit der in SEQ ID NO: 2 dargestellten Aminosäuresequenzen codieren und mindestens
30 50 % Homologie auf Aminosäureebene aufweisen, ohne daß die enzymatische Wirkung der Polypeptide wesentlich reduziert ist.
10. Transgener Organismus nach Anspruch 9, dadurch gekennzeichnet,
35 zeichnet, daß es sich bei dem Organismus um eine Pflanze oder Alge handelt.
11. Öl, Lipide oder Fettsäuren oder eine Fraktion davon, hergestellt durch das Verfahren nach einem der Ansprüche 1
40 bis 8.
12. Verwendung der Öl-, Lipid- oder Fettsäurezusammensetzung nach Anspruch 11 oder transgene Organismen nach Anspruch 9 in Futtermitteln, Nahrungsmitteln, Kosmetika oder Pharmazeutika.

SEQUENZPROTOKOLL

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4

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5

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6

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 00/06223

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/82 C12N9/02 C12N15/53 C12P7/64 C11C3/00
A01H5/00 A01H13/00 A01H15/00 A23L1/30 A23K1/16
A61K35/78

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, EMBASE, BIOSIS, MEDLINE, SCISEARCH, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	SPERLING, P. ET AL.: "A bifunctional delta-6-fatty acyl acetylenase/desaturase from the moss <i>Ceratodon purpureus</i> " EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 267, June 2000 (2000-06), pages 3801-3811, XP000941309 the whole document	1-4,7-11
X	GIRKE, T. ET AL.: "Identification of a novel delta-6-acyl-group desaturase by targeted gene disruption in <i>Physcomitrella patens</i> " THE PLANT JOURNAL, vol. 15, no. 1, July 1998 (1998-07), pages 39-48, XP000881712 cited in the application	1-4,7-11
Y	the whole document	5,6

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

A document defining the general state of the art which is not considered to be of particular relevance

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O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

G document member of the same patent family

Date of the actual completion of the international search

9 November 2000

Date of mailing of the international search report

24/11/2000

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 00/06223

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 46764 A (CALGENE LLC) 22 November 1998 (1998-11-22) cited in the application	11, 12
Y	page 5, line 27 -page 6, line 17 page 8, line 19 -page 36, line 27; examples 6-8, 13, 14, 16 -----	1-10
X	WO 99 27111 A (UNIVERSITY OF BRISTOL) 3 June 1999 (1999-06-03) cited in the application	11
Y	page 4, line 7 -page 9, line 28; examples 1, 2 -----	1-10
X	SAYANOVA, O. ET AL.: "Expression of a borage desaturase cDNA containing an N-terminal cytochrome b5 domain results in the accumulation of high levels of delta-6-desaturated fatty acids in transgenic tobacco" PROC.NATL.ACAD.SCI.USA, vol. 94, April 1997 (1997-04), pages 4211-4216, XP002099447 cited in the application	11
Y	the whole document -----	1-10
X	WO 96 21022 A (RHONE-POULENC AGROCHIMIE) 11 July 1996 (1996-07-11) cited in the application	11
Y	page 3, line 3 - line 23 page 5, line 16 -page 19, line 24; examples 6, 13, 14 -----	1-10

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 00/06223

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		CN 1252099 T	03-05-2000
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		US 5789220 A	04-08-1998



INTERNATIONALER REISEFORSCHENBERICHT

Intel  Aktenzeichen

PCT/EP 00/06223

A. KLASSIFIZIERUNG DES ANMELDUNGSGEGENSTANDES

IPK 7	C12N15/82	C12N9/02	C12N15/53	C12P7/64	C11C3/00
	A01H5/00	A01H13/00	A01H15/00	A23L1/30	A23K1/16
	A61K35/78				

Nach der Internationalen Patentklassifikation (IPK) oder nach der nationalen Klassifikation und der IPK

B. RECHERCHIERTE GEBIETE

Recherchierter Mindestprüfstoff (Klassifikationssystem und Klassifikationssymbole)

IPK 7 C12N C12P

Recherchierte aber nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Gebiete fallen

Während der internationalen Recherche konsultierte elektronische Datenbank (Name der Datenbank und evtl. verwendete Suchbegriffe)

EPO-Internal, WPI Data, PAJ, EMBASE, BIOSIS, MEDLINE, SCISEARCH, STRAND

C. ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie°	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
P,X	SPERLING, P. ET AL.: "A bifunctional delta-6-fatty acyl acetylenase/desaturase from thr moss <i>Ceratodon purpureus</i> " EUROPEAN JOURNAL OF BIOCHEMISTRY, Bd. 267, Juni 2000 (2000-06), Seiten 3801-3811, XP000941309 das ganze Dokument	1-4,7-11
X	--- GIRKE, T. ET AL.: "Identification of a novel delta-6-acyl-group desaturase by targeted gene disruption in <i>Physcomitrella patens</i> " THE PLANT JOURNAL, Bd. 15, Nr. 1, Juli 1998 (1998-07), Seiten 39-48, XP000881712 in der Anmeldung erwähnt	1-4,7-11
Y	das ganze Dokument ---	5,6
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Y	Seite 5, Zeile 27 -Seite 6, Zeile 17 Seite 8, Zeile 19 -Seite 36, Zeile 27; Beispiele 6-8,13,14,16 ----	1-10
X	WO 99 27111 A (UNIVERSITY OF BRISTOL) 3. Juni 1999 (1999-06-03) in der Anmeldung erwähnt	11
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Y	Seite 3, Zeile 3 - Zeile 23 Seite 5, Zeile 16 -Seite 19, Zeile 24; Beispiele 6,13,14 -----	1-10

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Angaben zu Veröffentlichungen, die zur selben Patentfamilie gehören

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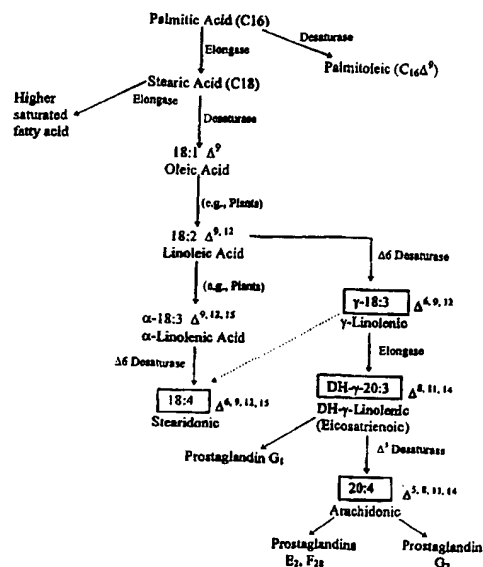
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(54) Title: METHODS AND COMPOSITIONS FOR SYNTHESIS OF LONG CHAIN POLYUNSATURATED FATTY ACIDS IN PLANTS

(57) Abstract

The present invention relates to compositions and methods for preparing polyunsaturated long chain fatty acids in plants, plant parts and plant cells, such as leaves, roots, fruits and seeds. Nucleic acid sequences and constructs encoding fatty acid desaturases, including Δ^5 -desaturases, Δ^6 -desaturases and Δ^{12} -desaturases, are used to generate transgenic plants, plant parts and cells which contain and express one or more transgenes encoding one or more desaturases. Expression of the desaturases with different substrate specificities in the plant system permit the large scale production of polyunsaturated long chain fatty acids such as docosahexaenoic acid, eicosapentaenoic acid, α -linolenic acid, gamma-linolenic acid, arachidonic acid and the like for modification of the fatty acid profile of plants, plant parts and tissues. Manipulation of the fatty acid profiles allows for the production of commercial quantities of novel plant oils and products.



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METHODS AND COMPOSITIONS FOR SYNTHESIS OF LONG CHAIN POLYUNSATURATED FATTY ACIDS IN PLANTS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of USSN 08/834,655, filed
5 April 11, 1997, and a continuation in part of USSN 08/833,610, filed April 11,
1997, USSN 08/834,033 filed April 11, 1997 and USSN 08/956,985 filed
October 24, 1997 which disclosures are incorporated herein by reference.

INTRODUCTION

Field of the Invention

10 This invention relates to modulating levels of enzymes and/or enzyme
components capable of altering the production of long chain polyunsaturated
fatty acids (PUFAS) in a host plant. The invention is exemplified by the
production of PUFAS in plants.

Background

15 Two main families of polyunsaturated fatty acids (PUFAs) are the $\omega 3$
fatty acids, exemplified by arachidonic acid, and the $\omega 6$ fatty acids, exemplified
by eicosapentaenoic acid. PUFAs are important components of the plasma
membrane of the cell, where they may be found in such forms as phospholipids.
PUFAs also serve as precursors to other molecules of importance in human
20 beings and animals, including the prostacyclins, leukotrienes and
prostaglandins. PUFAs are necessary for proper development, particularly in
the developing infant brain, and for tissue formation and repair.

Four major long chain PUFAs of importance include docosahexaenoic
acid (DHA) and eicosapentaenoic acid (EPA), which are primarily found in
25 different types of fish oil, gamma-linolenic acid (GLA), which is found in the
seeds of a number of plants, including evening primrose (*Oenothera biennis*),
borage (*Borago officinalis*) and black currants (*Ribes nigrum*), and stearidonic
acid (SDA), which is found in marine oils and plant seeds. Both GLA and
another important long chain PUFA, arachidonic acid (ARA), are found in

filamentous fungi. ARA can be purified from animal tissues including liver and adrenal gland.

For DHA, a number of sources exist for commercial production including a variety of marine organisms, oils obtained from cold water marine fish, and egg yolk fractions. For ARA, microorganisms including the genera *Mortierella*, *Entomophthora*, *Phytium* and *Porphyridium* can be used for commercial production. Commercial sources of SDA include the genera *Trichodesma* and *Echium*. Commercial sources of GLA include evening primrose, black currants and borage. However, there are several disadvantages associated with commercial production of PUFAs from natural sources. Natural sources of PUFAs, such as animals and plants, tend to have highly heterogeneous oil compositions. The oils obtained from these sources therefore can require extensive purification to separate out one or more desired PUFAs or to produce an oil which is enriched in one or more PUFA. Natural sources also are subject to uncontrollable fluctuations in availability. Fish stocks may undergo natural variation or may be depleted by overfishing. Fish oils have unpleasant tastes and odors, which may be impossible to economically separate from the desired product, and can render such products unacceptable as food supplements. Animal oils, and particularly fish oils, can accumulate environmental pollutants. Weather and disease can cause fluctuation in yields from both fish and plant sources. Cropland available for production of alternate oil-producing crops is subject to competition from the steady expansion of human populations and the associated increased need for food production on the remaining arable land. Crops which do produce PUFAs, such as borage, have not been adapted to commercial growth and may not perform well in monoculture. Growth of such crops is thus not economically competitive where more profitable and better established crops can be grown. Large scale fermentation of organisms such as *Mortierella* is also expensive. Natural animal tissues contain low amounts of ARA and are difficult to process. Microorganisms such as *Porphyridium* and *Mortierella* are difficult to cultivate on a commercial scale.

Dietary supplements and pharmaceutical formulations containing PUFAs can retain the disadvantages of the PUFA source. Supplements such as fish oil capsules can contain low levels of the particular desired component and thus require large dosages. High dosages result in ingestion of high levels of undesired components, including contaminants. Care must be taken in providing fatty acid supplements, as overaddition may result in suppression of endogenous biosynthetic pathways and lead to competition with other necessary fatty acids in various lipid fractions *in vivo*, leading to undesirable results. For example, Eskimos having a diet high in ω 3 fatty acids have an increased tendency to bleed (U.S. Pat. No. 4,874,603). Unpleasant tastes and odors of the supplements can make such regimens undesirable, and may inhibit compliance by the patient.

A number of enzymes are involved in PUFA biosynthesis. Linoleic acid (LA, 18:2 Δ 9, 12) is produced from oleic acid (18:1 Δ 9) by a Δ 12-desaturase. GLA (18:3 Δ 6, 9, 12) is produced from linoleic acid (LA, 18:2 Δ 9, 12) by a Δ 6-desaturase. ARA (20:4 Δ 5, 8, 11, 14) production from DGLA (20:3 Δ 8, 11, 14) is catalyzed by a Δ 5-desaturase. However, animals cannot desaturate beyond the Δ 9 position and therefore cannot convert oleic acid (18:1 Δ 9) into linoleic acid (18:2 Δ 9, 12). Likewise, α -linolenic acid (ALA, 18:3 Δ 9, 12, 15) cannot be synthesized by mammals. Other eukaryotes, including fungi and plants, have enzymes which desaturate at positions Δ 21 and Δ 15. The major poly-unsaturated fatty acids of animals therefore are either derived from diet and/or from desaturation and elongation of linoleic acid (18:2 Δ 9, 12) or α -linolenic acid (18:3 Δ 9, 12, 15).

Poly-unsaturated fatty acids are considered to be useful for nutritional, pharmaceutical, industrial, and other purposes. An expansive supply of poly-unsaturated fatty acids from natural sources and from chemical synthesis are not sufficient for commercial needs. Therefore it is of interest to obtain genetic material involved in PUFA biosynthesis from species that naturally produce these fatty acids and to express the isolated material alone or in combination in

a heterologous system which can be manipulated to allow production of commercial quantities of PUFAS.

The present invention is further directed to formulas, dietary supplements or dietary supplements in the form of a liquid or a solid containing
5 the long chain fatty acids of the invention. These formulas and supplements may be administered to a human or an animal.

The formulas and supplements of the invention may further comprise at least one macronutrient selected from the group consisting of coconut oil, soy oil, canola oil, mono- and diglycerides, glucose, edible lactose, electro dialysed
10 whey, electro dialysed skim milk, milk whey, soy protein, and other protein hydrolysates.

The formulas of the present invention may further include at least one vitamin selected from the group consisting of Vitamins A, C, D, E, and B complex; and at least one mineral selected from the group consisting of
15 calcium, magnesium, zinc, manganese, sodium, potassium, phosphorus, copper, chloride, iodine, selenium, and iron.

The present invention is further directed to a method of treating a patient having a condition caused by insufficient intake or production of polyunsaturated fatty acids comprising administering to the patient a dietary substitute of the
20 invention in an amount sufficient to effect treatment of the patient.

The present invention is further directed to cosmetic and pharmaceutical compositions of the material of the invention.

The present invention is further directed to transgenic oils in pharmaceutically acceptable carriers. The present invention is further directed
25 to nutritional supplements, cosmetic agents and infant formulae containing transgenic oils.

The present invention is further directed to a method for obtaining altered long chain polyunsaturated fatty acid biosynthesis comprising the steps of: growing a microbe having cells which contain a transgene which encodes a

transgene expression product which desaturates a fatty acid molecule at carbon 5,5 or 12 from the carboxyl end of said fatty acid molecule, wherein the transgene is operably associated with an expression control sequence, under conditions whereby the transgene is expressed, whereby long chain
5 polyunsaturated fatty acid biosynthesis in the cells is altered.

The present invention is further directed toward pharmaceutical compositions comprising at least one nutrient selected from the group consisting of a vitamin, a mineral, a carbohydrate, a sugar, an amino acid, a free fatty acid, a phospholipid, an antioxidant, and a phenolic compound.

10 Relevant Literature

Production of gamma-linolenic acid by a $\Delta 6$ -desaturase is described in USPN 5,552,306 and USPN 5,614,393. Production of 8, 11-eicosadienoic acid using *Mortierella alpina* is disclosed in USPN 5,376,541. Production of docosahexaenoic acid by dinoflagellates is described in USPN 5,407,957.
15 Cloning of a $\Delta 6$ -desaturase from borage is described in PCT publication WO 96/21022. Cloning of $\Delta 9$ -desaturases is described in the published patent applications PCT WO 91/13972, EP 0 550 162 A1, EP 0 561 569 A2, EP 0 644 263 A2, and EP 0 736 598 A1, and in USPN 5,057,419. Cloning of $\Delta 12$ -desaturases from various organisms is described in PCT publication WO
20 94/11516 and USPN 5,443,974. Cloning of $\Delta 15$ -desaturases from various organisms is described in PCT publication WO 93/11245. A $\Delta 6$ palmitoyl-acyl carrier protein desaturase from *Thumbergia alata* and its expression in *E. coli* is described in USPN 5,614,400. Expression of a soybean stearyl-ACP desaturase in transgenic soybean embryos using a 35S promoter is disclosed in USPN
25 5,443,974.

SUMMARY OF THE INVENTION

Novel compositions and methods are provided for preparation of poly-unsaturated long chain fatty acids and desaturases in plants and plant cells. The methods involve growing a host plant cell of interest transformed with an
30 expression cassette functional in a host plant cell, the expression cassette

comprising a transcriptional and translational initiation regulatory region, joined in reading frame 5' to a DNA sequence encoding a desaturase polypeptide capable of modulating the production of PUFAs. Expression of the desaturase polypeptide provides for an alteration in the PUFA profile of host plant cells as a result of altered concentrations of enzymes involved in PUFA biosynthesis. Of particular interest is the selective control of PUFA production in plant tissues and/or plant parts such as leaves, roots, fruits and seeds. The invention finds use for example in the large scale production of DHA, EPA, ARA, and GLA and for modification of the fatty acid profile of edible plant tissues and/or plant parts.

The present invention further includes a purified nucleotide sequence or polypeptide sequence that is substantially related or homologous to the nucleotide and peptide sequences presented in SEQ ID NO:1 - SEQ ID NO:52. The present invention is further directed to methods of using the sequences presented in SEQ ID NO:1 to SEQ ID NO:40 as probes to identify related sequences, as components of expression systems and as components of systems useful for producing transgenic oil.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows possible pathways for the synthesis of arachidonic acid (20:4 Δ 5, 8, 11, 14) and stearidonic acid (18:4 Δ 6, 9, 12, 15) from palmitic acid (C_{16}) from a variety of organisms, including algae, *Mortierella* and humans. These PUFAs can serve as precursors to other molecules important for humans and other animals, including prostacyclins, leukotrienes, and prostaglandins, some of which are shown.

Figure 2 shows possible pathways for production of PUFAs in addition to ARA, including EPA and DHA, again compiled from a variety of organisms.

Figure 3A-E shows the DNA sequence (SEQ ID NO:1) of the *Mortierella alpina* Δ 6 desaturase and the deduced amino acid sequence (SEQ ID NO:2).

Figure 4 shows an alignment of the *Mortierella alpina* $\Delta 6$ desaturase amino acid sequence with other $\Delta 6$ desaturases and related sequences (SEQ ID NOS:7, 8, 9, 10, 11, 12 and 13).

Figure 5A-D shows the DNA sequence of the *Mortierella alpina* $\Delta 12$ desaturase (SEQ ID NO:3) and the deduced amino acid sequence (SEQ ID NO:4)

Figure 6 shows the deduced amino acid sequence (SEQ ID NO:14) of the PCR fragment (see Example 1).

Figure 7A-D shows the DNA sequence of the *Mortierella alpina* $\Delta 5$ desaturase (SEQ ID NO:5).

Figure 8 shows alignments of the protein sequence of the $\Delta 5$ desaturase (SEQ ID NO:6) with $\Delta 6$ desaturases and related sequences (SEQ ID NOS:15, 16, 17, 18).

Figure 9 shows alignments of the protein sequence of the Ma 29 and contig 253538a.

Figure 10 shows alignments of the protein sequence of Ma 524 and contig 253538a.

BRIEF DESCRIPTION OF THE SEQUENCE LISTINGS

SEQ ID NO:1 shows the DNA sequence of the *Mortierella alpina* $\Delta 6$ desaturase.

SEQ ID NO:2 shows the amino acid sequence of the *Mortierella alpina* $\Delta 6$ desaturase.

SEQ ID NO:3 shows the DNA sequence of the *Mortierella alpina* $\Delta 12$ desaturase.

SEQ ID NO:4 shows the amino acid sequence of the *Mortierella alpina* $\Delta 12$ desaturase.

SEQ ID NO:5 shows the DNA sequence of the *Mortierella alpina* $\Delta 5$ desaturase.

SEQ ID NO:6 shows the amino acid sequence *Mortierella alpina* $\Delta 5$ desaturase.

5 SEQ ID NO:7 - SEQ ID NO:13 show amino acid sequences that relate to *Mortierella alpina* $\Delta 6$ desaturase.

SEQ ID NO:14 shows an amino acid sequence of a PCR fragment of Example 1.

10 SEQ ID NO:15 - SEQ ID NO:18 show amino acid sequences that relate to *Mortierella alpina* $\Delta 5$ and $\Delta 6$ desaturases.

SEQ ID NO:19 - SEQ ID NO:30 show PCR primer sequences.

SEQ ID NO:31 - SEQ ID NO:37 show human nucleotide sequences.

SEQ ID NO:38 - SEQ ID NO:44 show human peptide sequences.

15 SEQ ID NO:45 - SEQ ID NO:46 show the nucleotide and amino acid sequence of a *Dictyostelium discoideum* desaturase.

SEQ ID NO:47 - SEQ ID NO:50 show the nucleotide and deduced amino acid sequence of a *Schizochytrium* cDNA clone.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

20 In order to ensure a complete understanding of the invention, the following definitions are provided:

$\Delta 5$ -Desaturase: $\Delta 5$ desaturase is an enzyme which introduces a double bond between carbons 5 and 6 from the carboxyl end of a fatty acid molecule.

$\Delta 6$ -Desaturase: $\Delta 6$ -desaturase is an enzyme which introduces a double bond between carbons 6 and 7 from the carboxyl end of a fatty acid molecule.

25 **$\Delta 9$ -Desaturase:** $\Delta 9$ -desaturase is an enzyme which introduces a double bond between carbons 9 and 10 from the carboxyl end of a fatty acid molecule.

Δ 12-Desaturase: Δ 12-desaturase is an enzyme which introduces a double bond between carbons 12 and 13 from the carboxyl end of a fatty acid molecule.

- 5 **Fatty Acids:** Fatty acids are a class of compounds containing a long hydrocarbon chain and a terminal carboxylate group. Fatty acids include the following:

Fatty Acid		
12:0	lauric acid	
16:0	palmitic acid	
16:1	palmitoleic acid	
18:0	stearic acid	
18:1	oleic acid	Δ 9-18:1
18:2 Δ 5,9	taxoleic acid	Δ 5,9-18:2
18:2 Δ 6,9	6,9-octadecadienoic acid	Δ 6,9-18:2
18:2	linoleic acid	Δ 9,12-18:2 (LA)
18:3 Δ 6,9,12	gamma-linolenic acid	Δ 6,9,12-18:3 (GLA)
18:3 Δ 5,9,12	pinolenic acid	Δ 5,9,12-18:3
18:3	alpha-linolenic acid	Δ 9,12,15-18:3 (ALA)
18:4	stearidonic acid	Δ 6,9,12,15-18:4 (SDA)
20:0	Arachidic acid	
20:1	Eicosenic Acid	
22:0	behehic acid	
22:1	erucic acid	
22:2	Docasadienoic acid	
20:4 ω 6	arachidonic acid	Δ 5,8,11,14-20:4 (ARA)
20:3 ω 6	ω 6-eicosatrienoic dihomo-gamma linolenic	Δ 8,11,14-20:3 (DGLA)
20:5 ω 3	Eicosapentanoic (Timnodonic acid)	Δ 5,8,11,14,17-20:5 (EPA)
20:3 ω 3	ω 3-eicosatrienoic	Δ 11,16,17-20:3
20:4 ω 3	ω 3-eicosatetraenoic	Δ 8,11,14,17-20:4
22:5 ω 3	Docosapentaenoic	Δ 7,10,13,16,19-22:5 (ω 3DPA)
22:6 ω 3	Docosahexaenoic (cervonic acid)	Δ 4,7,10,13,16,19-22:6 (DHA)
24:0	Lignoceric acid	

Taking into account these definitions, the present invention is directed to novel DNA sequences, DNA constructs, methods and compositions are provided which permit modification of the poly-unsaturated long chain fatty acid content of plant cells. Plant cells are transformed with an expression cassette

5 comprising a DNA encoding a polypeptide capable of increasing the amount of one or more PUFA in a plant cell. Desirably, integration constructs may be prepared which provide for integration of the expression cassette into the genome of a host cell. Host cells are manipulated to express a sense or antisense DNA encoding a polypeptide(s) that has desaturase activity. By

10 "desaturase" is intended a polypeptide which can desaturate one or more fatty acids to produce a mono- or poly-unsaturated fatty acid or precursor thereof of interest. By "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification, for example, glycosylation or phosphorylation. The substrate(s) for the expressed enzyme may be produced

15 by the host cell or may be exogenously supplied.

To achieve expression in a host cell, the transformed DNA is operably associated with transcriptional and translational initiation and termination regulatory regions that are functional in the host cell. Constructs comprising the gene to be expressed can provide for integration into the genome of the host cell

20 or can autonomously replicate in the host cell. For production of linoleic acid (LA), the expression cassettes generally used include a cassette which provides for $\Delta 12$ desaturase activity, particularly in a host cell which produces or can take up oleic acid. For production of ALA, the expression cassettes generally used include a cassette which provides for $\Delta 15$ or $\omega 3$ desaturase activity,

25 particularly in a host cell which produces or can take up LA. For production of GLA or SDA, the expression cassettes generally used include a cassette which provides for $\Delta 6$ desaturase activity, particularly in a host cell which produces or can take up LA or ALA, respectively. Production of $\omega 6$ -type unsaturated fatty acids, such as LA or GLA, is favored in a plant capable of producing ALA by

30 inhibiting the activity of a $\Delta 15$ or $\omega 3$ type desaturase; this is accomplished by providing an expression cassette for an antisense $\Delta 15$ or $\omega 3$ transcript, or by

disrupting a $\Delta 15$ or $\omega 3$ desaturase gene. Similarly, production of LA or ALA is favored in a plant having $\Delta 6$ desaturase activity by providing an expression cassette for an antisense $\Delta 6$ transcript, or by disrupting a $\Delta 6$ desaturase gene. Production of oleic acid likewise is favored in a plant having $\Delta 12$ desaturase activity by providing an expression cassette for an antisense $\Delta 12$ transcript, or by disrupting a $\Delta 12$ desaturase gene. For production of ARA, the expression cassette generally used provides for $\Delta 5$ desaturase activity, particularly in a host cell which produces or can take up DGLA. Production of $\omega 6$ -type unsaturated fatty acids, such as ARA, is favored in a plant capable of producing ALA by inhibiting the activity of a $\Delta 15$ or $\omega 3$ type desaturase; this is accomplished by providing an expression cassette for an antisense $\Delta 15$ or $\omega 3$ transcript, or by disrupting a $\Delta 15$ or $\omega 3$ desaturase gene.

TRANSGENIC PLANT PRODUCTION OF FATTY ACIDS

Transgenic plant production of PUFAs offers several advantages over purification from natural sources such as fish or plants. Production of fatty acids from recombinant plants provides the ability to alter the naturally occurring plant fatty acid profile by providing new synthetic pathways in the host or by suppressing undesired pathways, thereby increasing levels of desired PUFAs, or conjugated forms thereof, and decreasing levels of undesired PUFAs. Production of fatty acids in transgenic plants also offers the advantage that expression of desaturase genes in particular tissues and/or plant parts means that greatly increased levels of desired PUFAs in those tissues and/or parts can be achieved, making recovery from those tissues more economical. For example, the desired PUFAs can be expressed in seed; methods of isolating seed oils are well established. In addition to providing a source for purification of desired PUFAs, seed oil components can be manipulated through expression of desaturase genes, either alone or in combination with other genes such as elongases, to provide seed oils having a particular PUFA profile in concentrated form. The concentrated seed oils then can be added to animal milks and/or synthetic or semi-synthetic milks to serve as infant formulas where human

nursing is impossible or undesired, or in cases of malnourishment or disease in both adults and infants.

For production of PUFAs, depending upon the host cell, the availability of substrate, and the desired end product(s), several polypeptides, particularly desaturases, are of interest including those polypeptides which catalyze the conversion of stearic acid to oleic acid, LA to GLA, of ALA to SDA, of oleic acid to LA, or of LA to ALA, which includes enzymes which desaturate at the $\Delta 6$, $\Delta 9$, $\Delta 12$, $\Delta 15$ or $\omega 3$ positions. Considerations for choosing a specific polypeptide having desaturase activity include the pH optimum of the polypeptide, whether the polypeptide is a rate limiting enzyme or a component thereof, whether the desaturase used is essential for synthesis of a desired polyunsaturated fatty acid, and/or co-factors required by the polypeptide. The expressed polypeptide preferably has parameters compatible with the biochemical environment of its location in the host cell. For example, the polypeptide may have to compete for substrate with other enzymes in the host cell. Analyses of the K_m and specific activity of the polypeptide in question therefore are considered in determining the suitability of a given polypeptide for modifying PUFA production in a given host cell. The polypeptide used in a particular situation therefore is one which can function under the conditions present in the intended host cell but otherwise can be any polypeptide having desaturase activity which has the desired characteristic of being capable of modifying the relative production of a desired PUFA. A scheme for the synthesis of arachidonic acid (20:4 $\Delta 5$, 8, 11, 14) from palmitic acid (C_{16}) is shown in Figure 1. A key enzyme in this pathway is a $\Delta 5$ -desaturase which converts DH- γ -linolenic acid (DGLA, eicosatrienoic acid) to ARA. Conversion of α -linolenic acid (ALA) to stearidonic acid by a $\Delta 6$ -desaturase is also shown. Production of PUFAs in addition to ARA, including EPA and DHA is shown in Figure 2. A key enzyme in the synthesis of arachidonic acid (20:4 $\Delta 5$, 8, 11, 14) from stearic acid (C_{18}) is a $\Delta 6$ -desaturase which converts the linoleic acid into γ -linolenic acid. Conversion of α -linolenic acid (ALA) to stearidonic acid by a $\Delta 6$ -desaturase also is shown. For production of ARA, the DNA sequence

used encodes a polypeptide having $\Delta 5$ desaturase activity. In particular instances, this can be coupled with an expression cassette which provides for production of a polypeptide having $\Delta 6$ desaturase activity and, optionally, a transcription cassette providing for production of antisense sequences to a $\Delta 15$ transcription product. The choice of combination of cassettes used depends in part on the PUFA profile of the host cell. Where the host cell $\Delta 5$ -desaturase activity is limiting, overexpression of $\Delta 5$ desaturase alone generally will be sufficient to provide for enhanced ARA production.

SOURCES OF POLYPEPTIDES HAVING DESATURASE ACTIVITY

As sources of polypeptides having desaturase activity and oligonucleotides encoding such polypeptides are organisms which produce a desired poly-unsaturated fatty acid. As an example, microorganisms having an ability to produce ARA can be used as a source of $\Delta 5$ -desaturase genes; microorganisms which GLA or SDA can be used as a source of $\Delta 6$ -desaturase and/or $\Delta 12$ -desaturase genes. Such microorganisms include, for example, those belonging to the genera *Mortierella*, *Conidiobolus*, *Pythium*, *Phytophthora*, *Penicillium*, *Porphyridium*, *Coidosporium*, *Mucor*, *Fusarium*, *Aspergillus*, *Rhodotorula*, and *Entomophthora*. Within the genus *Porphyridium*, of particular interest is *Porphyridium cruentum*. Within the genus *Mortierella*, of particular interest are *Mortierella elongata*, *Mortierella exigua*, *Mortierella hygrophila*, *Mortierella ramanniana*, var. *angulispora*, and *Mortierella alpina*. Within the genus *Mucor*, of particular interest are *Mucor circinelloides* and *Mucor javanicus*.

DNAs encoding desired desaturases can be identified in a variety of ways. As an example, a source of the desired desaturase, for example genomic or cDNA libraries from *Mortierella*, is screened with detectable enzymatically- or chemically-synthesized probes, which can be made from DNA, RNA, or non-naturally occurring nucleotides, or mixtures thereof. Probes may be enzymatically synthesized from DNAs of known desaturases for normal or

reduced-stringency hybridization methods. Oligonucleotide probes also can be used to screen sources and can be based on sequences of known desaturases, including sequences conserved among known desaturases, or on peptide sequences obtained from the desired purified protein. Oligonucleotide probes
5 based on amino acid sequences can be degenerate to encompass the degeneracy of the genetic code, or can be biased in favor of the preferred codons of the source organism. Oligonucleotides also can be used as primers for PCR from reverse transcribed mRNA from a known or suspected source; the PCR product can be the full length cDNA or can be used to generate a probe to obtain the
10 desired full length cDNA. Alternatively, a desired protein can be entirely sequenced and total synthesis of a DNA encoding that polypeptide performed.

Once the desired genomic or cDNA has been isolated, it can be sequenced by known methods. It is recognized in the art that such methods are subject to errors, such that multiple sequencing of the same region is routine and
15 is still expected to lead to measurable rates of mistakes in the resulting deduced sequence, particularly in regions having repeated domains, extensive secondary structure, or unusual base compositions, such as regions with high GC base content. When discrepancies arise, resequencing can be done and can employ special methods. Special methods can include altering sequencing conditions
20 by using: different temperatures; different enzymes; proteins which alter the ability of oligonucleotides to form higher order structures; altered nucleotides such as ITP or methylated dGTP; different gel compositions, for example adding formamide; different primers or primers located at different distances from the problem region; or different templates such as single stranded DNAs.
25 Sequencing of mRNA can also be employed.

For the most part, some or all of the coding sequence for the polypeptide having desaturase activity is from a natural source. In some situations, however, it is desirable to modify all or a portion of the codons, for example, to enhance expression, by employing host preferred codons. Host preferred
30 codons can be determined from the codons of highest frequency in the proteins expressed in the largest amount in a particular host species of interest. Thus, the

coding sequence for a polypeptide having desaturase activity can be synthesized in whole or in part. All or portions of the DNA also can be synthesized to remove any destabilizing sequences or regions of secondary structure which would be present in the transcribed mRNA. All or portions of the DNA also can be synthesized to alter the base composition to one more preferable in the desired host cell. Methods for synthesizing sequences and bringing sequences together are well established in the literature. *In vitro* mutagenesis and selection, site-directed mutagenesis, or other means can be employed to obtain mutations of naturally occurring desaturase genes to produce a polypeptide having desaturase activity *in vivo* with more desirable physical and kinetic parameters for function in the host cell, such as a longer half-life or a higher rate of production of a desired polyunsaturated fatty acid.

Desirable cDNAs have less than 60% A+T composition, preferably less than 50% A+T composition. On a localized scale of a sliding window of 20 base pairs, it is preferable that there are no localized regions of the cDNA with greater than 75% A+T composition; with a window of 60 base pairs, it is preferable that there are no localized regions of the cDNA with greater than 60%, more preferably no localized regions with greater than 55% A+T composition.

Mortierella alpina Desaturases

Of particular interest are the *Mortierella alpina* $\Delta 5$ -desaturase, $\Delta 6$ -desaturase and $\Delta 12$ -desaturase. The $\Delta 5$ -desaturase has 446 amino acids; the amino acid sequence is shown in Figure 7. The gene encoding the *Mortierella alpina* $\Delta 5$ -desaturase can be expressed in transgenic microorganisms to effect greater synthesis of ARA from DGLA. Other DNAs which are substantially identical in sequence to the *Mortierella alpina* $\Delta 5$ -desaturase DNA, or which encode polypeptides which are substantially identical in sequence to the *Mortierella alpina* $\Delta 5$ -desaturase polypeptide, also can be used. The *Mortierella alpina* $\Delta 6$ -desaturase, has 457 amino acids and a predicted molecular weight of 51.8 kD; the amino acid sequence is shown in Figure 3.

The gene encoding the *Mortierella alpina* $\Delta 6$ -desaturase can be expressed in transgenic plants or animals to effect greater synthesis of GLA from linoleic acid or of stearidonic acid (SDA) from ALA. Other DNAs which are substantially identical in sequence to the *Mortierella alpina* $\Delta 6$ -desaturase DNA, or which encode polypeptides which are substantially identical in sequence to the *Mortierella alpina* $\Delta 6$ -desaturase polypeptide, also can be used.

The *Mortierella alpina* $\Delta 12$ -desaturase has the amino acid sequence shown in Figure 5. The gene encoding the *Mortierella alpina* $\Delta 12$ -desaturase can be expressed in transgenic plants to effect greater synthesis of LA from oleic acid. Other DNAs which are substantially identical to the *Mortierella alpina* $\Delta 12$ -desaturase DNA, or which encode polypeptides which are substantially identical to the *Mortierella alpina* $\Delta 12$ -desaturase polypeptide, also can be used.

By substantially identical in sequence is intended an amino acid sequence or nucleic acid sequence exhibiting in order of increasing preference at least 60%, 80%, 90% or 95% homology to the *Mortierella alpina* $\Delta 5$ -desaturase amino acid sequence or nucleic acid sequence encoding the amino acid sequence. For polypeptides, the length of comparison sequences generally is at least 16 amino acids, preferably at least 20 amino acids, or most preferably 35 amino acids. For nucleic acids, the length of comparison sequences generally is at least 50 nucleotides, preferably at least 60 nucleotides, and more preferably at least 75 nucleotides, and most preferably, 110 nucleotides. Homology typically is measured using sequence analysis software, for example, the Sequence Analysis software package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wisconsin 53705, MEGAlign (DNASar, Inc., 1228 S. Park St., Madison, Wisconsin 53715), and MacVector (Oxford Molecular Group, 2105 S. Bascom Avenue, Suite 200, Campbell, California 95008). Such software matches similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications. Conservative substitutions typically include substitutions within the following groups: glycine and alanine;

valine, isoleucine and leucine; aspartic acid, glutamic acid, asparagine, and glutamine; serine and threonine; lysine and arginine; and phenylalanine and tyrosine. Substitutions may also be made on the basis of conserved hydrophobicity or hydrophilicity (Kyte and Doolittle, *J. Mol. Biol.* 157: 105-132, 1982), or on the basis of the ability to assume similar polypeptide secondary structure (Chou and Fasman, *Adv. Enzymol.* 47: 45-148, 1978).

Other Desaturases

Encompassed by the present invention are related desaturases from the same or other organisms. Such related desaturases include variants of the disclosed $\Delta 5$ -, $\Delta 6$ - and $\Delta 12$ -desaturases that occur naturally within the same or different species of *Mortierella*, as well as homologues of the disclosed $\Delta 5$ -desaturase from other species and evolutionarily related protein having desaturase activity. Also included are desaturases which, although not substantially identical to the *Mortierella alpina* $\Delta 5$ -desaturase, desaturate a fatty acid molecule at carbon 5, 6 or 12, respectively, from the carboxyl end of a fatty acid molecule. Related desaturases can be identified by their ability to function substantially the same as the disclosed desaturases; that is, are still able to effectively convert DGLA to ARA, LA to GLA, ALA to SDA or oleic acid to LA. Related desaturases also can be identified by screening sequence databases for sequences homologous to the disclosed desaturase, by hybridization of a probe based on the disclosed desaturase to a library constructed from the source organism, or by RT-PCR using mRNA from the source organism and primers based on the disclosed desaturase. Such desaturases includes those from humans, *Dictyostelium discoideum* and *Phaeodactylum tricornutum*.

The regions of a desaturase polypeptide important for desaturase activity can be determined through routine mutagenesis, expression of the resulting mutant polypeptides and determination of their activities. Mutants may include deletions, insertions and point mutations, or combinations thereof. A typical functional analysis begins with deletion mutagenesis to determine the N- and C-terminal limits of the protein necessary for function, and then internal deletions,

insertions or point mutants are made to further determine regions necessary for function. Other techniques such as cassette mutagenesis or total synthesis also can be used. Deletion mutagenesis is accomplished, for example, by using exonucleases to sequentially remove the 5' or 3' coding regions. Kits are
5 available for such techniques. After deletion, the coding region is completed by ligating oligonucleotides containing start or stop codons to the deleted coding region after 5' or 3' deletion, respectively. Alternatively, oligonucleotides encoding start or stop codons are inserted into the coding region by a variety of methods including site-directed mutagenesis, mutagenic PCR or by ligation
10 onto DNA digested at existing restriction sites. Internal deletions can similarly be made through a variety of methods including the use of existing restriction sites in the DNA, by use of mutagenic primers via site directed mutagenesis or mutagenic PCR. Insertions are made through methods such as linker-scanning mutagenesis, site-directed mutagenesis or mutagenic PCR. Point mutations are
15 made through techniques such as site-directed mutagenesis or mutagenic PCR.

Chemical mutagenesis can also be used for identifying regions of a desaturase polypeptide important for activity. A mutated construct is expressed, and the ability of the resulting altered protein to function as a desaturase is assayed. Such structure-function analysis can determine which regions may be
20 deleted, which regions tolerate insertions, and which point mutations allow the mutant protein to function in substantially the same way as the native desaturase. All such mutant proteins and nucleotide sequences encoding them are within the scope of the present invention.

EXPRESSION OF DESATURASE GENES

25 Once the DNA encoding a desaturase polypeptide has been obtained, it is placed in a vector capable of replication in a host cell, or is propagated *in vitro* by means of techniques such as PCR or long PCR. Replicating vectors can include plasmids, phage, viruses, cosmids and the like. Desirable vectors include those useful for mutagenesis of the gene of interest or for expression of
30 the gene of interest in host cells. The technique of long PCR has made *in vitro* propagation of large constructs possible, so that modifications to the gene of

interest, such as mutagenesis or addition of expression signals, and propagation of the resulting constructs can occur entirely *in vitro* without the use of a replicating vector or a host cell.

For expression of a desaturase polypeptide, functional transcriptional
5 and translational initiation and termination regions are operably linked to the DNA encoding the desaturase polypeptide. Transcriptional and translational initiation and termination regions are derived from a variety of nonexclusive sources, including the DNA to be expressed, genes known or suspected to be capable of expression in the desired system, expression vectors, chemical
10 synthesis, or from an endogenous locus in a host cell. Expression in a plant tissue and/or plant part presents certain efficiencies, particularly where the tissue or part is one which is easily harvested, such as seed, leaves, fruits, flowers, roots, etc. Expression can be targeted to that location within the plant by using specific regulatory sequences, such as those of USPN 5,463,174,
15 USPN 4,943,674, USPN 5,106,739, USPN 5,175,095, USPN 5,420,034, USPN 5,188,958, and USPN 5,589,379. Alternatively, the expressed protein can be an enzyme which produces a product which may be incorporated, either directly or upon further modifications, into a fluid fraction from the host plant. In the present case, expression of desaturase genes, or antisense desaturase transcripts,
20 can alter the levels of specific PUFAs, or derivatives thereof, found in plant parts and/or plant tissues. The $\Delta 5$ -desaturase polypeptide coding region is expressed either by itself or with other genes, in order to produce tissues and/or plant parts containing higher proportions of desired PUFAs or in which the PUFA composition more closely resembles that of human breast milk (Prieto *et al.*, PCT publication WO 95/24494). The termination region can be derived
25 from the 3' region of the gene from which the initiation region was obtained or from a different gene. A large number of termination regions are known to and have been found to be satisfactory in a variety of hosts from the same and different genera and species. The termination region usually is selected more as
30 a matter of convenience rather than because of any particular property.

The choice of a host cell is influenced in part by the desired PUFA profile of the transgenic cell, and the native profile of the host cell. As an example, for production of linoleic acid from oleic acid, the DNA sequence used encodes a polypeptide having $\Delta 12$ desaturase activity, and for production of GLA from linoleic acid, the DNA sequence used encodes a polypeptide having $\Delta 6$ desaturase activity. Use of a host cell which expresses $\Delta 12$ desaturase activity and lacks or is depleted in $\Delta 15$ desaturase activity, can be used with an expression cassette which provides for overexpression of $\Delta 6$ desaturase alone generally is sufficient to provide for enhanced GLA production in the transgenic cell. Where the host cell expresses $\Delta 9$ desaturase activity, expression of both a $\Delta 12$ - and a $\Delta 6$ -desaturase can provide for enhanced GLA production. In particular instances where expression of $\Delta 6$ desaturase activity is coupled with expression of $\Delta 12$ desaturase activity, it is desirable that the host cell naturally have, or be mutated to have, low $\Delta 15$ desaturase activity. Alternatively, a host cell for $\Delta 6$ desaturase expression may have, or be mutated to have, high $\Delta 12$ desaturase activity.

Expression in a host cell can be accomplished in a transient or stable fashion. Transient expression can occur from introduced constructs which contain expression signals functional in the host cell, but which constructs do not replicate and rarely integrate in the host cell, or where the host cell is not proliferating. Transient expression also can be accomplished by inducing the activity of a regulatable promoter operably linked to the gene of interest, although such inducible systems frequently exhibit a low basal level of expression. Stable expression can be achieved by introduction of a construct that can integrate into the host genome or that autonomously replicates in the host cell. Stable expression of the gene of interest can be selected for through the use of a selectable marker located on or transfected with the expression construct, followed by selection for cells expressing the marker. When stable expression results from integration, integration of constructs can occur randomly within the host genome or can be targeted through the use of constructs containing regions of homology with the host genome sufficient to

target recombination with the host locus. Where constructs are targeted to an endogenous locus, all or some of the transcriptional and translational regulatory regions can be provided by the endogenous locus.

When increased expression of the desaturase polypeptide in the source
5 plant is desired, several methods can be employed. Additional genes encoding the desaturase polypeptide can be introduced into the host organism. Expression from the native desaturase locus also can be increased through homologous recombination, for example by inserting a stronger promoter into the host genome to cause increased expression, by removing destabilizing
10 sequences from either the mRNA or the encoded protein by deleting that information from the host genome, or by adding stabilizing sequences to the mRNA (*see* USPN 4,910,141 and USPN 5,500,365.)

When it is desirable to express more than one different gene, appropriate regulatory regions and expression methods, introduced genes can be propagated
15 in the host cell through use of replicating vectors or by integration into the host genome. Where two or more genes are expressed from separate replicating vectors, it is desirable that each vector has a different means of replication. Each introduced construct, whether integrated or not, should have a different means of selection and should lack homology to the other constructs to maintain
20 stable expression and prevent reassortment of elements among constructs. Judicious choices of regulatory regions, selection means and method of propagation of the introduced construct can be experimentally determined so that all introduced genes are expressed at the necessary levels to provide for synthesis of the desired products.

25 Constructs comprising the gene of interest may be introduced into a host cell by standard techniques. These techniques include transfection, infection, bolistic impact, electroporation, microinjection, scraping, or any other method which introduces the gene of interest into the host cell (*see* USPN 4,743,548, USPN 4,795,855, USPN 5,068,193, USPN 5,188,958, USPN 5,463,174, USPN
30 5,565,346 and USPN 5,565,347). For convenience, a host cell which has been manipulated by any method to take up a DNA sequence or construct will be

referred to as "transformed" or "recombinant" herein. The subject host will have at least have one copy of the expression construct and may have two or more, depending upon whether the gene is integrated into the genome, amplified, or is present on an extrachromosomal element having multiple copy
5 numbers.

The transformed host cell can be identified by selection for a marker contained on the introduced construct. Alternatively, a separate marker construct may be introduced with the desired construct, as many transformation techniques introduce many DNA molecules into host cells. Typically,
10 transformed hosts are selected for their ability to grow on selective media. Selective media may incorporate an antibiotic or lack a factor necessary for growth of the untransformed host, such as a nutrient or growth factor. An introduced marker gene therefor may confer antibiotic resistance, or encode an essential growth factor or enzyme, and permit growth on selective media when
15 expressed in the transformed host cell. Desirably, resistance to kanamycin and the amino glycoside G418 are of interest (see USPN 5,034,322). Selection of a transformed host can also occur when the expressed marker protein can be detected, either directly or indirectly. The marker protein may be expressed alone or as a fusion to another protein. The marker protein can be detected by
20 its enzymatic activity; for example β galactosidase can convert the substrate X-gal to a colored product, and luciferase can convert luciferin to a light-emitting product. The marker protein can be detected by its light-producing or modifying characteristics; for example, the green fluorescent protein of *Aequorea victoria* fluoresces when illuminated with blue light. Antibodies can
25 be used to detect the marker protein or a molecular tag on, for example, a protein of interest. Cells expressing the marker protein or tag can be selected, for example, visually, or by techniques such as FACS or panning using antibodies.

The PUFAs produced using the subject methods and compositions may
30 be found in the host plant tissue and/or plant part as free fatty acids or in conjugated forms such as acylglycerols, phospholipids, sulfolipids or

glycolipids, and may be extracted from the host cell through a variety of means well-known in the art. Such means may include extraction with organic solvents, sonication, supercritical fluid extraction using for example carbon dioxide, and physical means such as presses, or combinations thereof. Of particular interest is extraction with hexane or methanol and chloroform. Where desirable, the aqueous layer can be acidified to protonate negatively charged moieties and thereby increase partitioning of desired products into the organic layer. After extraction, the organic solvents can be removed by evaporation under a stream of nitrogen. When isolated in conjugated forms, the products are enzymatically or chemically cleaved to release the free fatty acid or a less complex conjugate of interest, and are then subjected to further manipulations to produce a desired end product. Desirably, conjugated forms of fatty acids are cleaved with potassium hydroxide.

PURIFICATION OF FATTY ACIDS

If further purification is necessary, standard methods can be employed. Such methods include extraction, treatment with urea, fractional crystallization, HPLC, fractional distillation, silica gel chromatography, high speed centrifugation or distillation, or combinations of these techniques. Protection of reactive groups, such as the acid or alkenyl groups, may be done at any step through known techniques, for example alkylation or iodination. Methods used include methylation of the fatty acids to produce methyl esters. Similarly, protecting groups may be removed at any step. Desirably, purification of fractions containing ARA, DHA and EPA is accomplished by treatment with urea and/or fractional distillation.

USES OF FATTY ACIDS

The uses of the fatty acids of subject invention are several. Probes based on the DNAs of the present invention may find use in methods for isolating related molecules or in methods to detect organisms expressing desaturases. When used as probes, the DNAs or oligonucleotides need to be detectable. This is usually accomplished by attaching a label either at an internal site, for

- example via incorporation of a modified residue, or at the 5' or 3' terminus. Such labels can be directly detectable, can bind to a secondary molecule that is detectably labeled, or can bind to an unlabelled secondary molecule and a detectably labeled tertiary molecule; this process can be extended as long as is practical to achieve a satisfactorily detectable signal without unacceptable levels of background signal. Secondary, tertiary, or bridging systems can include use of antibodies directed against any other molecule, including labels or other antibodies, or can involve any molecules which bind to each other, for example a biotin-streptavidin/avidin system. Detectable labels typically include radioactive isotopes, molecules which chemically or enzymatically produce or alter light, enzymes which produce detectable reaction products, magnetic molecules, fluorescent molecules or molecules whose fluorescence or light-emitting characteristics change upon binding. Examples of labelling methods can be found in USPN 5,011,770. Alternatively, the binding of target molecules can be directly detected by measuring the change in heat of solution on binding of probe to target via isothermal titration calorimetry, or by coating the probe or target on a surface and detecting the change in scattering of light from the surface produced by binding of target or probe, respectively, as may be done with the BIAcore system.
- PUFAs of the subject invention produced by recombinant means find applications in a wide variety of areas. Supplementation of humans or animals with PUFAs in various forms can result in increased levels not only of the added PUFAs, but of their metabolic progeny as well. For example, where the inherent $\Delta 6$ -desaturase pathway is dysfunctional in an individual, treatment with GLA can result not only in increased levels of GLA, but also of downstream products such as ARA and prostaglandins (see Figure 1). Complex regulatory mechanisms can make it desirable to combine various PUFAs, or to add different conjugates of PUFAs, in order to prevent, control or overcome such mechanisms to achieve the desired levels of specific PUFAs in an individual.
- PUFAs, or derivatives thereof, made by the disclosed method can be used as dietary supplements, particularly in infant formulas, for patients

undergoing intravenous feeding or for preventing or treating malnutrition. Particular fatty acids such as EPA are used to alter the composition of infant formulas to better replicate the PUFA composition of human breast milk. The predominant triglyceride in human milk has been reported to be 1,3-di-oleoyl-2-palmitoyl, with 2-palmitoyl glycerides reported as better absorbed than 2-oleoyl or 2-lineoyl glycerides (USPN 4,876,107). Typically, human breast milk has a fatty acid profile comprising from about 0.15 % to about 0.36 % as DHA, from about 0.03 % to about 0.13 % as EPA, from about 0.30 % to about 0.88 % as ARA, from about 0.22 % to about 0.67 % as DGLA, and from about 0.27 % to about 1.04 % as GLA. A preferred ratio of GLA:DGLA:ARA in infant formulas is from about 1:1:4 to about 1:1:1, respectively. Amounts of oils providing these ratios of PUFA can be determined without undue experimentation by one of skill in the art. PUFAs, or host cells containing them, also can be used as animal food supplements to alter an animal's tissue or milk fatty acid composition to one more desirable for human or animal consumption.

NUTRITIONAL COMPOSITIONS

The present invention also includes nutritional compositions. Such compositions, for purposes of the present invention, include any food or preparation for human consumption including for enteral or parenteral consumption, which when taken into the body (a) serve to nourish or build up tissues or supply energy and/or (b) maintain, restore or support adequate nutritional status or metabolic function.

The nutritional composition of the present invention comprises at least one oil or acid produced in accordance with the present invention and may either be in a solid or liquid form. Additionally, the composition may include edible macronutrients, vitamins and minerals in amounts desired for a particular use. The amount of such ingredients will vary depending on whether the composition is intended for use with normal, healthy infants, children or adults having specialized needs such as those which accompany certain metabolic conditions (e.g., metabolic disorders).

Examples of macronutrients which may be added to the composition include but are not limited to edible fats, carbohydrates and proteins. Examples of such edible fats include but are not limited to coconut oil, soy oil, and mono- and diglycerides. Examples of such carbohydrates include but are not limited to
5 glucose, edible lactose and hydrolyzed starch. Additionally, examples of proteins which may be utilized in the nutritional composition of the invention include but are not limited to soy proteins, electrodialysed whey, electrodialysed skim milk, milk whey, or the hydrolysates of these proteins.

With respect to vitamins and minerals, the following may be added to
10 the nutritional compositions of the present invention: calcium, phosphorus, potassium, sodium, chloride, magnesium, manganese, iron, copper, zinc, selenium, iodine, and Vitamins A, E, D, C, and the B complex. Other such vitamins and minerals may also be added.

The components utilized in the nutritional compositions of the present
15 invention will be of semi-purified or purified origin. By semi-purified or purified is meant a material which has been prepared by purification of a natural material or by synthesis.

Examples of nutritional compositions of the present invention include but are not limited to infant formulas, dietary supplements, and rehydration
20 compositions. Nutritional compositions of particular interest include but are not limited to those utilized for enteral and parenteral supplementation for infants, specialist infant formulae, supplements for the elderly, and supplements for those with gastrointestinal difficulties and/or malabsorption.

Nutritional Compositions

25 A typical nutritional composition of the present invention will contain edible macronutrients, vitamins and minerals in amounts desired for a particular use. The amounts of such ingredients will vary depending on whether the formulation is intended for use with normal, healthy individuals temporarily exposed to stress, or to subjects having specialized needs due to certain chronic
30 or acute disease states (e.g., metabolic disorders). It will be understood by

persons skilled in the art that the components utilized in a nutritional formulation of the present invention are of semi-purified or purified origin. By semi-purified or purified is meant a material that has been prepared by purification of a natural material or by synthesis. These techniques are well known in the art (See, e.g., Code of Federal Regulations for Food Ingredients and Food Processing; Recommended Dietary Allowances, 10th Ed., National Academy Press, Washington, D.C., 1989).

In a preferred embodiment, a nutritional formulation of the present invention is an enteral nutritional product, more preferably an adult or child enteral nutritional product. Accordingly in a further aspect of the invention, a nutritional formulation is provided that is suitable for feeding adults or children who are experiencing stress. The formula comprises, in addition to the PUFAs of the invention; macronutrients, vitamins and minerals in amounts designed to provide the daily nutritional requirements of adults.

The macronutritional components include edible fats, carbohydrates and proteins. Exemplary edible fats are coconut oil, soy oil, and mono- and diglycerides and the PUFA oils of this invention. Exemplary carbohydrates are glucose, edible lactose and hydrolyzed cornstarch. A typical protein source would be soy protein, electrodialysed whey or electrodialysed skim milk or milk whey, or the hydrolysates of these proteins, although other protein sources are also available and may be used. These macronutrients would be added in the form of commonly accepted nutritional compounds in amount equivalent to those present in human milk or an energy basis, i.e., on a per calorie basis.

Methods for formulating liquid and enteral nutritional formulas are well known in the art and are described in detail in the examples.

The enteral formula can be sterilized and subsequently utilized on a ready-to-feed (RTF) basis or stored in a concentrated liquid or a powder. The powder can be prepared by spray drying the enteral formula prepared as indicated above, and the formula can be reconstituted by rehydrating the concentrate. Adult and infant nutritional formulas are well known in the art and commercially available (e.g., Similac®, Ensure®, Jevity® and Alimentum®).

from Ross Products Division, Abbott Laboratories). An oil or acid of the present invention can be added to any of these formulas in the amounts described below.

5 The energy density of the nutritional composition when in liquid form, can typically range from about 0.6 Kcal to 3 Kcal per ml. When in solid or powdered form, the nutritional supplement can contain from about 1.2 to more than 9 Kcals per gm, preferably 3 to 7 Kcals per gm. In general, the osmolality of a liquid product should be less than 700 mOsm and more preferably less than 660 mOsm.

10 The nutritional formula would typically include vitamins and minerals, in addition to the PUFAs of the invention, in order to help the individual ingest the minimum daily requirements for these substances. In addition to the PUFAs listed above, it may also be desirable to supplement the nutritional composition with zinc, copper, and folic acid in addition to antioxidants. It is believed that
15 these substances will also provide a boost to the stressed immune system and thus will provide further benefits to the individual. The presence of zinc, copper or folic acid is optional and is not required in order to gain the beneficial effects on immune suppression. Likewise a pharmaceutical composition can be supplemented with these same substances as well.

20 In a more preferred embodiment, the nutritional contains, in addition to the antioxidant system and the PUFA component, a source of carbohydrate wherein at least 5 weight % of said carbohydrate is an indigestible oligosaccharide. In yet a more preferred embodiment, the nutritional composition additionally contains protein, taurine and carnitine.

25 The PUFAs, or derivatives thereof, made by the disclosed method can be used as dietary substitutes, or supplements, particularly infant formulas, for patients undergoing intravenous feeding or for preventing or treating malnutrition. Typically, human breast milk has a fatty acid profile comprising from about 0.15 % to about 0.36 % as DHA, from about 0.03 % to about 0.13 %
30 as EPA, from about 0.30 % to about 0.88 % as ARA, from about 0.22 % to about 0.67 % as DGLA, and from about 0.27 % to about 1.04 % as GLA.

Additionally, the predominant triglyceride in human milk has been reported to be 1,3-di-oleoyl-2-palmitoyl, with 2-palmitoyl glycerides reported as better absorbed than 2-oleoyl or 2-lineoyl glycerides (USPN 4,876,107). Thus, fatty acids such as ARA, DGLA, GLA and/or EPA produced by the invention can be used to alter the composition of infant formulas to better replicate the PUFA composition of human breast milk. In particular, an oil composition for use in a pharmacologic or food supplement, particularly a breast milk substitute or supplement, will preferably comprise one or more of ARA, DGLA and GLA. More preferably the oil will comprise from about 0.3 to 30% ARA, from about 0.2 to 30% DGLA, and from about 0.2 to about 30% GLA.

In addition to the concentration, the ratios of ARA, DGLA and GLA can be adapted for a particular given end use. When formulated as a breast milk supplement or substitute, an oil composition which contains two or more of ARA, DGLA and GLA will be provided in a ratio of about 1:19:30 to about 6:1:0.2, respectively. For example, the breast milk of animals can vary in ratios of ARA:DGLA:DGL ranging from 1:19:30 to 6:1:0.2, which includes intermediate ratios which are preferably about 1:1:1, 1:2:1, 1:1:4. When produced together in a host cell, adjusting the rate and percent of conversion of a precursor substrate such as GLA and DGLA to ARA can be used to precisely control the PUFA ratios. For example, a 5% to 10% conversion rate of DGLA to ARA can be used to produce an ARA to DGLA ratio of about 1:19, whereas a conversion rate of about 75% to 80% can be used to produce an ARA to DGLA ratio of about 6:1. Therefore, whether in a cell culture system or in a host animal, regulating the timing, extent and specificity of desaturase expression as described can be used to modulate the PUFA levels and ratios. Depending on the expression system used, e.g., cell culture or an animal expressing oil(s) in its milk, the oils also can be isolated and recombined in the desired concentrations and ratios. Amounts of oils providing these ratios of PUFA can be determined following standard protocols. PUFAs, or host cells containing them, also can be used as animal food supplements to alter an animal's tissue or milk fatty acid composition to one more desirable for human or animal consumption.

For dietary supplementation, the purified PUFAs, or derivatives thereof, may be incorporated into cooking oils, fats or margarines formulated so that in normal use the recipient would receive the desired amount. The PUFAs may also be incorporated into infant formulas, nutritional supplements or other food products, and may find use as anti-inflammatory or cholesterol lowering agents.

Pharmaceutical Compositions

The present invention also encompasses a pharmaceutical composition comprising one or more of the acids and/or resulting oils produced in accordance with the methods described herein. More specifically, such a pharmaceutical composition may comprise one or more of the acids and/or oils as well as a standard, well-known, non-toxic pharmaceutically acceptable carrier, adjuvant or vehicle such as, for example, phosphate buffered saline, water, ethanol, polyols, vegetable oils, a wetting agent or an emulsion such as a water/oil emulsion. The composition may be in either a liquid or solid form.

For example, the composition may be in the form of a tablet, capsule, ingestible liquid or powder, injectible, or topical ointment or cream.

Possible routes of administration include, for example, oral, rectal and parenteral. The route of administration will, of course, depend upon the desired effect. For example, if the composition is being utilized to treat rough, dry, or aging skin, to treat injured or burned skin, or to treat skin or hair affected by a disease or condition, it may perhaps be applied topically.

The dosage of the composition to be administered to the patient may be determined by one of ordinary skill in the art and depends upon various factors such as weight of the patient, age of the patient, immune status of the patient, etc.

With respect to form, the composition may be, for example, a solution, a dispersion, a suspension, an emulsion or a sterile powder which is then reconstituted.

Additionally, the composition of the present invention may be utilized for cosmetic purposes. It may be added to pre-existing cosmetic compositions such that a mixture is formed or may be used as a sole composition.

Pharmaceutical compositions may be utilized to administer the PUFA
5 component to an individual. Suitable pharmaceutical compositions may comprise physiologically acceptable sterile aqueous or non-aqueous solutions, dispersions, suspensions or emulsions and sterile powders for reconstitution into sterile solutions or dispersions for ingestion. Examples of suitable aqueous and non-aqueous carriers, diluents, solvents or vehicles include water, ethanol,
10 polyols (propyleneglycol, polyethyleneglycol, glycerol, and the like), suitable mixtures thereof, vegetable oils (such as olive oil) and injectable organic esters such as ethyl oleate. Proper fluidity can be maintained, for example, by the maintenance of the required particle size in the case of dispersions and by the use of surfactants. It may also be desirable to include isotonic agents, for
15 example sugars, sodium chloride and the like. Besides such inert diluents, the composition can also include adjuvants, such as wetting agents, emulsifying and suspending agents, sweetening, flavoring and perfuming agents.

Suspensions, in addition to the active compounds, may contain suspending agents, as for example, ethoxylated isostearyl alcohols,
20 polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth or mixtures of these substances, and the like.

Solid dosage forms such as tablets and capsules can be prepared using techniques well known in the art. For example, PUFAs of the invention can be
25 tableted with conventional tablet bases such as lactose, sucrose, and cornstarch in combination with binders such as acacia, cornstarch or gelatin, disintegrating agents such as potato starch or alginic acid and a lubricant such as stearic acid or magnesium stearate. Capsules can be prepared by incorporating these excipients into a gelatin capsule along with the antioxidants and the PUFA
30 component. The amount of the antioxidants and PUFA component that should

be incorporated into the pharmaceutical formulation should fit within the guidelines discussed above.

As used in this application, the term "treat" refers to either preventing, or reducing the incidence of, the undesired occurrence. For example, to treat
5 immune suppression refers to either preventing the occurrence of this suppression or reducing the amount of such suppression. The terms "patient" and "individual" are being used interchangeably and both refer to an animal. The term "animal" as used in this application refers to any warm-blooded mammal including, but not limited to, dogs, humans, monkeys, and apes. As
10 used in the application the term "about" refers to an amount varying from the stated range or number by a reasonable amount depending upon the context of use. Any numerical number or range specified in the specification should be considered to be modified by the term about.

"Dose" and "serving" are used interchangeably and refer to the amount
15 of the nutritional or pharmaceutical composition ingested by the patient in a single setting and designed to deliver effective amounts of the antioxidants and the structured triglyceride. As will be readily apparent to those skilled in the art, a single dose or serving of the liquid nutritional powder should supply the amount of antioxidants and PUFAs discussed above. The amount of the dose or
20 serving should be a volume that a typical adult can consume in one sitting. This amount can vary widely depending upon the age, weight, sex or medical condition of the patient. However as a general guideline, a single serving or dose of a liquid nutritional produce should be considered as encompassing a volume from 100 to 600 ml, more preferably from 125 to 500 ml and most
25 preferably from 125 to 300 ml.

The PUFAs of the present invention may also be added to food even when supplementation of the diet is not required. For example, the composition may be added to food of any type including but not limited to margarines, modified butters, cheeses, milk, yogurt, chocolate, candy, snacks, salad oils,
30 cooking oils, cooking fats, meats, fish and beverages.

Pharmaceutical Applications

For pharmaceutical use (human or veterinary), the compositions are generally administered orally but can be administered by any route by which they may be successfully absorbed, e.g., parenterally (i.e. subcutaneously, intramuscularly or intravenously), rectally or vaginally or topically, for example, as a skin ointment or lotion. The PUFAs of the present invention may be administered alone or in combination with a pharmaceutically acceptable carrier or excipient. Where available, gelatin capsules are the preferred form of oral administration. Dietary supplementation as set forth above also can provide an oral route of administration. The unsaturated acids of the present invention may be administered in conjugated forms, or as salts, esters, amides or prodrugs of the fatty acids. Any pharmaceutically acceptable salt is encompassed by the present invention; especially preferred are the sodium, potassium or lithium salts. Also encompassed are the N-alkylpolyhydroxamine salts, such as N-methyl glucamine, found in PCT publication WO 96/33155. The preferred esters are the ethyl esters. As solid salts, the PUFAs also can be administered in tablet form. For intravenous administration, the PUFAs or derivatives thereof may be incorporated into commercial formulations such as Intralipids. The typical normal adult plasma fatty acid profile comprises 6.64 to 9.46% of ARA, 1.45 to 3.11% of DGLA, and 0.02 to 0.08% of GLA. These PUFAs or their metabolic precursors can be administered, either alone or in mixtures with other PUFAs, to achieve a normal fatty acid profile in a patient. Where desired, the individual components of formulations may be individually provided in kit form, for single or multiple use. A typical dosage of a particular fatty acid is from 0.1 mg to 20 g, or even 100 g daily, and is preferably from 10 mg to 1, 2, 5 or 10 g daily as required, or molar equivalent amounts of derivative forms thereof. Parenteral nutrition compositions comprising from about 2 to about 30 weight percent fatty acids calculated as triglycerides are encompassed by the present invention; preferred is a composition having from about 1 to about 25 weight percent of the total PUFA composition as GLA (USPN 5,196,198). Other vitamins, and particularly fat-soluble vitamins such as vitamin A, D, E and L-carnitine can optionally be included. Where desired, a

preservative such as α tocopherol may be added, typically at about 0.1% by weight.

Suitable pharmaceutical compositions may comprise physiologically acceptable sterile aqueous or non-aqueous solutions, dispersions, suspensions or emulsions and sterile powders for reconstitution into sterile injectible solutions or dispersions. Examples of suitable aqueous and non-aqueous carriers, diluents, solvents or vehicles include water, ethanol, polyols (propyleneglyol, polyethyleneglycol, glycerol, and the like), suitable mixtures thereof, vegetable oils (such as olive oil) and injectable organic esters such as ethyl oleate. Proper fluidity can be maintained, for example, by the maintenance of the required particle size in the case of dispersions and by the use of surfactants. It may also be desirable to include isotonic agents, for example sugars, sodium chloride and the like. Besides such inert diluents, the composition can also include adjuvants, such as wetting agents, emulsifying and suspending agents, sweetening, flavoring and perfuming agents.

Suspensions in addition to the active compounds, may contain suspending agents, as for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, or mixtures of these substances and the like.

An especially preferred pharmaceutical composition contains diacetyltartaric acid esters of mono- and diglycerides dissolved in an aqueous medium or solvent. Diacetyltartaric acid esters of mono- and diglycerides have an HLB value of about 9-12 and are significantly more hydrophilic than existing antimicrobial lipids that have HLB values of 2-4. Those existing hydrophobic lipids cannot be formulated into aqueous compositions. As disclosed herein, those lipids can now be solubilized into aqueous media in combination with diacetyltartaric acid esters of mono- and diglycerides. In accordance with this embodiment, diacetyltartaric acid esters of mono- and diglycerides (e.g., DATEM-C12:0) is melted with other active antimicrobial lipids (e.g., 18:2 and 12:0 monoglycerides) and mixed to obtain a homogeneous mixture.

Homogeneity allows for increased antimicrobial activity. The mixture can be completely dispersed in water. This is not possible without the addition of diacetyltartaric acid esters of mono- and diglycerides and premixing with other monoglycerides prior to introduction into water. The aqueous composition can
5 then be admixed under sterile conditions with physiologically acceptable diluents, preservatives, buffers or propellants as may be required to form a spray or inhalant.

The present invention also encompasses the treatment of numerous disorders with fatty acids. Supplementation with PUFAs of the present
10 invention can be used to treat restenosis after angioplasty. Symptoms of inflammation, rheumatoid arthritis, and asthma and psoriasis can be treated with the PUFAs of the present invention. Evidence indicates that PUFAs may be involved in calcium metabolism, suggesting that PUFAs of the present invention may be used in the treatment or prevention of osteoporosis and of
15 kidney or urinary tract stones.

The PUFAs of the present invention can be used in the treatment of cancer. Malignant cells have been shown to have altered fatty acid compositions; addition of fatty acids has been shown to slow their growth and cause cell death, and to increase their susceptibility to chemotherapeutic agents.
20 GLA has been shown to cause reexpression on cancer cells of the E-cadherin cellular adhesion molecules, loss of which is associated with aggressive metastasis. Clinical testing of intravenous administration of the water soluble lithium salt of GLA to pancreatic cancer patients produced statistically significant increases in their survival. PUFA supplementation may also be
25 useful for treating cachexia associated with cancer.

The PUFAs of the present invention can also be used to treat diabetes (USPN 4,826,877; Horrobin *et al.*, Am. J. Clin. Nutr. Vol. 57 (Suppl.), 732S-737S). Altered fatty acid metabolism and composition has been demonstrated in diabetic animals. These alterations have been suggested to be involved in
30 some of the long-term complications resulting from diabetes, including retinopathy, neuropathy, nephropathy and reproductive system damage.

Primrose oil, which contains GLA, has been shown to prevent and reverse diabetic nerve damage.

The PUFAs of the present invention can be used to treat eczema, reduce blood pressure and improve math scores. Essential fatty acid deficiency has
5 been suggested as being involved in eczema, and studies have shown beneficial effects on eczema from treatment with GLA. GLA has also been shown to reduce increases in blood pressure associated with stress, and to improve performance on arithmetic tests. GLA and DGLA have been shown to inhibit platelet aggregation, cause vasodilation, lower cholesterol levels and inhibit
10 proliferation of vessel wall smooth muscle and fibrous tissue (Brenner *et al.*, Adv. Exp. Med. Biol. Vol. 83, p. 85-101, 1976). Administration of GLA or DGLA, alone or in combination with EPA, has been shown to reduce or prevent gastro-intestinal bleeding and other side effects caused by non-steroidal anti-inflammatory drugs (USPN 4,666,701). GLA and DGLA have also been shown
15 to prevent or treat endometriosis and premenstrual syndrome (USPN 4,758,592) and to treat myalgic encephalomyelitis and chronic fatigue after viral infections (USPN 5,116,871).

Further uses of the PUFAs of this invention include use in treatment of AIDS, multiple sclerosis, acute respiratory syndrome, hypertension and
20 inflammatory skin disorders. The PUFAs of the inventions also can be used for formulas for general health as well as for geriatric treatments.

Veterinary Applications

It should be noted that the above-described pharmaceutical and nutritional compositions may be utilized in connection with animals, as well as
25 humans, as animals experience many of the same needs and conditions as human. For example, the oil or acids of the present invention may be utilized in animal feed supplements or as animal feed substitutes.

The following examples are presented by way of illustration, not of limitation.

Examples

- Example 1 Isolation of $\Delta 5$ Desaturase Nucleotide Sequence from *Mortierella alpina*
- 5 Example 2 Isolation of $\Delta 6$ Desaturase Nucleotide Sequence from *Mortierella alpina*
- Example 3 Identification of $\Delta 6$ Desaturases Homologues to the *Mortierella alpina* Δ Desaturase
- Example 4 Isolation of D-12 Desaturase Nucleotide Sequence from *Mortierella alpina*
- 10 Example 5 Isolation of Cytochrome b5 Reductase Nucleotide Sequence from *Mortierella alpina*
- Example 6 Expression of *M. alpina* Desaturase Clones in Baker's Yeast
- Example 7 Fatty Acid Analysis of Leaves from Ma29 Transgenic *Brassica* Plants
- 15 Example 8 Expression of *M. alpina* $\Delta 6$ Desaturase in *Brassica napus*
- Example 9 Expression of *M. alpina* $\Delta 12$ desaturase in *Brassica napus*
- 20 Example 10 Simultaneous expression of *M. alpina* $\Delta 6$ and $\Delta 12$ desaturases in *Brassica napus*
- Example 11 Simultaneous expression of *M. alpina* $\Delta 5$ and $\Delta 6$ desaturases in *Brassica napus*
- Example 12 Simultaneous expression of *M. alpina* $\Delta 5$, $\Delta 6$ and $\Delta 12$ desaturases in *Brassica napus*
- 25 Example 13 Stereospecific Distribution of $\Delta 6$ -Desaturated Oils
- Example 14 Fatty Acid Compositions of Transgenic Plants

Example 15 Combined Expression of $\Delta 6$ and $\Delta 12$ Desaturases in *B. napus* Achieved by Crossing

Example 16 Expression of *M. alpina* desaturases in soybean

Example 17 Human Desaturase Gene Sequences

5

Example 1

Isolation of a $\Delta 5$ -desaturase Nucleotide Sequence from *Mortierella alpina*

Mortierella alpina produces arachidonic acid (ARA, 20:4) from the precursor 20:3 by a $\Delta 5$ -desaturase. A nucleotide sequence encoding the $\Delta 5$ -desaturase from *Mortierella alpina* (see Figure 7) was obtained through PCR
10 amplification using *M. alpina* 1st strand cDNA and degenerate oligonucleotide primers corresponding to amino acid sequences conserved between $\Delta 6$ -desaturases from *Synechocystis* and *Spirulina*. The procedure used was as follows:

Total RNA was isolated from a 3 day old PUFA-producing culture of
15 *Mortierella alpina* using the protocol of Hoge *et al.* (1982) *Experimental Mycology* 6:225-232. The RNA was used to prepare double-stranded cDNA using BRL's lambda-ZipLox system, following the manufacturer's instructions. Several size fractions of the *M. alpina* cDNA were packaged separately to yield libraries with different average-sized inserts. The "full-length" library contains
20 approximately 3×10^6 clones with an average insert size of 1.77 kb. The "sequencing-grade" library contains approximately 6×10^5 clones with an average insert size of 1.1 kb.

5 μ g of total RNA was reverse transcribed using BRL Superscript RTase and the primer TSyn 5'-CAAGCTTCTGCAGGAGCTCTTTTTTTTTTTTTTTT-
25 3' (SEQ ID NO:19.) Degenerate oligonucleotides were designed to regions conserved between the two cyanobacterial $\Delta 6$ -desaturase sequences. The specific primers used were:

D6DESAT-F3 (SEQ ID NO:20)

5'-CUACUACUACUACAYCAYACOTAYACOAAYAT-3'

D6DESAT-R3 (SEQ ID NO:21)

5'-CAUCAUCAUCAUOGGAAOARRTGRTG-3'

- 5 where Y=C+T, R=A+G, and O=I+C. PCR amplification was carried out in a 25µl volume containing: template derived from 40 ng total RNA, 2 pM each primer, 200 µM each deoxyribonucleotide triphosphate, 60 mM Tris-Cl, pH 8.5, 15 mM (NH₄)₂SO₄, 2 mM MgCl₂. Samples were subjected to an initial desaturation step of 95 degrees (all temperatures Celsius) for 5 minutes, then
- 10 held at 72 degrees while 0.2 U of Taq polymerase were added. PCR thermocycling conditions were as follows: 94 degrees for 1 min., 45 degrees for 1.5 min., 72 degrees for 2 min. PCR was continued for 35 cycles. PCR using these primers on the *M. alpina* first-strand cDNA produced a 550 bp reaction product. Comparison of the deduced amino acid sequence of the *M.*
- 15 *alpina* PCR fragment revealed regions of homology with Δ6-desaturases (see Figure 4). However, there was only about 28% identity over the region compared. The deduced amino acid sequence is presented in SEQ ID NO:14.

- The PCR product was used as a probe to isolate corresponding cDNA clones from a *M. alpina* library. The longest cDNA clone, Ma29, was
- 20 designated pCGN5521 and has been completely sequenced on both strands. The cDNA is contained as a 1481 bp insert in the vector pZL1 (Bethesda Research Laboratories) and, beginning with the first ATG, contains an open reading frame encoding 446 amino acids. The reading frame contains the sequence deduced from the PCR fragment. The sequence of the cDNA insert
- 25 was found to contain regions of homology to Δ6-desaturases (see Figure 8). For example, three conserved "histidine boxes" (that have been observed in other membrane-bound desaturases (Okuley *et al.*, (1994) *The Plant Cell* 6:147-158)) were found to be present in the *Mortierella* sequence at amino acid positions 171-175, 207-212, and 387-391 (see Figure 5A-5D). However, the typical
- 30 "HXXHH" amino acid motif for the third histidine box for the *Mortierella*

desaturase was found to be QXXHH. The amino-terminus of the encoded protein, showed significant homology to cytochrome b5 proteins. Thus, the *Mortierella* cDNA clone appears to represent a fusion between a cytochrome b5 and a fatty acid desaturase. Since cytochrome b5 is believed to function as the electron donor for membrane-bound desaturase enzymes, it is possible that the N-terminal cytochrome b5 domain of this desaturase protein is involved in its function. This may be advantageous when expressing the desaturase in heterologous systems for PUFA production.

Example 2

10 Isolation of $\Delta 6$ Desaturase Nucleotide Sequence from *Mortierella alpina*

A nucleic acid sequence from a partial cDNA clone, Ma524, encoding a $\Delta 6$ fatty acid desaturase from *Mortierella alpina* was obtained by random sequencing of clones from the *M. alpina* cDNA library described in Example 1. cDNA-containing plasmids were excised as follows:

15 Five μ l of phage were combined with 100 μ l of *E. coli* DH10B(ZIP) grown in ECLB plus 10 μ g/ml kanamycin, 0.2% maltose, and 10 mM $MgSO_4$ and incubated at 37 degrees for 15 minutes. 0.9 ml SOC was added and 100 μ l of the bacteria immediately plated on each of 10 ECLB + 50 μ g Pen plates. No 45 minute recovery time was needed. The plates were incubated overnight at 37

20 degrees. Colonies were picked into ECLB + 50 μ g Pen media for overnight cultures to be used for making glycerol stocks and miniprep DNA. An aliquot of the culture used for the miniprep is stored as a glycerol stock. Plating on ECLB + 50 μ g Pen/ml resulted in more colonies and a greater proportion of colonies containing inserts than plating on 100 μ g/ml Pen.

25 Random colonies were picked and plasmid DNA purified using Qiagen miniprep kits. DNA sequence was obtained from the 5' end of the cDNA insert and compared to the databases using the BLAST algorithm. Ma524 was identified as a putative $\Delta 6$ desaturase based on DNA sequence homology to previously identified $\Delta 6$ desaturases. A full-length cDNA clone was isolated

from the *M. alpina* library. The abundance of this clone appears to be slightly (2X) less than Ma29. Ma524 displays significant homology to a portion of a *Caenorhabditis elegans* cosmid, WO6D2.4, a cytochrome b5/desaturase fusion protein from sunflower, and the two $\Delta 6$ desaturases in the public databanks
5 those from *Synechocystis* and *Spirulina*.

In addition, Ma524 shows significant homology to the borage $\Delta 6$ -desaturase sequence (PCT publication WO 96/21022). Ma524 thus appears to encode a $\Delta 6$ -desaturase that is related to the borage and algal $\Delta 6$ -desaturases. It should be noted that, although the amino acid sequences of Ma524 and the
10 borage $\Delta 6$ are similar, the base composition of the cDNAs is quite different: the borage cDNA has an overall base composition of 60 % A+T, with some regions exceeding 70 %, while Ma524 has an average of 44 % A+T base composition, with no regions exceeding 60 %. This may have implications for expressing the cDNAs in microorganisms or animals which favor different base compositions.
15 It is known that poor expression of recombinant genes can occur when the host has a very different base composition from that of the introduced gene. Speculated mechanisms for such poor expression include decreased stability or translatability of the mRNA.

Example 3

20 Identification of $\Delta 6$ -desaturases Homologous to the *Mortierella alpina* $\Delta 6$ -desaturase

Nucleic acid sequences that encode putative $\Delta 6$ -desaturases were identified through a BLASTX search of the est databases through NCBI using the Ma524 amino acid sequence. Several sequences showed significant
25 homology. In particular, the deduced amino acid sequence of two *Arabidopsis thaliana* sequences, (accession numbers F13728 and T42806) showed homology to two different regions of the deduced amino acid sequence of Ma524. The following PCR primers were designed: ATTS4723-FOR (complementary to F13728) 5'-CUACUACUACUAGGAGTCCTCTA
30 CGGTGTTTTG, SEQ ID NO:22, and T42806-REV (complementary to

T42806) 5' CAUCAUCAUATGATGCTCAAGCTGAACTG, SEQ ID NO:23. Five µg of total RNA isolated from developing siliques of *Arabidopsis thaliana* was reverse transcribed using BRL Superscript RTase and the primer TSyn 5'-CCAAGCTTCTGCAGGAGCTCTTTTTTTTTTTTTTTT-3', (SEQ ID NO:24). PCR was carried out in a 50 µl volume containing: template derived from 25 ng total RNA, 2 pM each primer, 200 µM each deoxyribonucleotide triphosphate, 60 mM Tris-Cl, pH 8.5, 15 mM (NH₄)₂SO₄, 2 mM MgCl₂, 0.2 U Taq Polymerase. Cycle conditions were as follows: 94 degrees for 30 sec., 50 degrees for 30 sec., 72 degrees for 30 sec. PCR was continued for 35 cycles followed by an additional extension at 72 degrees for 7 minutes. PCR resulted in a fragment of ~750 base pairs which was subsequently subcloned, named 12-5, and sequenced. Each end of this fragment corresponds to the *Arabidopsis* est from which the PCR primers were derived. This is the sequence named 12-5. The deduced amino acid sequence of 12-5 is compared to that of Ma524 and ests from human (W28140), mouse (W53753), and *C. elegans* (R05219) in Figure 4. Based on homology, these sequences represent desaturase polypeptides. The full-length genes can be cloned using probes based on the est sequences. The genes can then be placed in expression vectors and expressed in host cells and their specific Δ6- or other desaturase activity can be determined as described below.

Example 4

Isolation of Δ-12 Desaturase Nucleotide Sequence from *Mortierella alpina*

Based on the fatty acids it accumulates, *Mortierella alpina* has an ω6 type desaturase. The ω6 desaturase is responsible for the production of linoleic acid (18:2) from oleic acid (18:1). Linoleic acid (18:2) is a substrate for a Δ6 desaturase. This experiment was designed to determine if *Mortierella alpina* has a Δ12-desaturase polypeptide, and if so, to identify the corresponding nucleotide sequence. A random colony from the *M. alpina* sequencing grade library, Ma648, was sequenced and identified as a putative desaturase based on DNA sequence homology to previously identified desaturases, as described for

Ma524 (*see* Example 2). The deduced amino acid sequence from the 5' end of the Ma648 cDNA displays significant homology to soybean microsomal $\omega 6$ ($\Delta 12$) desaturase (accession #L43921) as well as castor bean oleate 12-hydroxylase (accession #U22378). In addition, homology is observed to a
5 variety of other $\omega 6$ ($\Delta 12$) and $\omega 3$ ($\Delta 15$) fatty acid desaturase sequences.

Example 5

Isolation of Cytochrome b5 Reductase Nucleotide Sequence from *Mortierella alpina*

A nucleic acid sequence encoding a cytochrome b5 reductase from
10 *Mortierella alpina* was obtained as follows. A cDNA library was constructed based on total RNA isolated from *Mortierella alpina* as described in Example 1. DNA sequence was obtained from the 5' and 3' ends of one of the clones, M12-27. A search of public databanks with the deduced amino acid sequence of the
15 3' end of M12-27 (*see* Figure 5) revealed significant homology to known cytochrome b5 reductase sequences. Specifically, over a 49 amino acid region, the *Mortierella* clone shares 55% identity (73% homology) with a cytochrome b5 reductase from pig (*see* Figure 4).

Example 6

Expression of *M. alpina* Desaturase Clones in Baker's Yeast Yeast Transformation

Lithium acetate transformation of yeast was performed according to standard protocols (*Methods in Enzymology*, Vol. 194, p. 186-187, 1991). Briefly, yeast were grown in YPD at 30°C. Cells were spun down, resuspended in TE, spun down again, resuspended in TE containing 100 mM lithium acetate,
25 spun down again, and resuspended in TE/lithium acetate. The resuspended yeast were incubated at 30°C for 60 minutes with shaking. Carrier DNA was added, and the yeast were aliquoted into tubes. Transforming DNA was added, and the tubes were incubated for 30 min. at 30°C. PEG solution (35% (w/v) PEG 4000, 100 mM lithium acetate, TE pH7.5) was added followed by a 50

min. incubation at 30°C. A 5 min. heat shock at 42°C was performed, the cells were pelleted, washed with TE, pelleted again and resuspended in TE. The resuspended cells were then plated on selective media.

Desaturase Expression in Transformed Yeast

5 cDNA clones from *Mortierella alpina* were screened for desaturase activity in baker's yeast. A canola $\Delta 15$ -desaturase (obtained by PCR using 1st strand cDNA from *Brassica napus* cultivar 212/86 seeds using primers based on the published sequence (Arondel *et al. Science* 258:1353-1355)) was used as a positive control. The $\Delta 15$ -desaturase gene and the gene from cDNA clone
10 Ma29 was put in the expression vector pYES2 (Invitrogen), resulting in plasmids pCGR-2 and pCGR-4, respectively. These plasmids were transfected into *S. cerevisiae* yeast strain 334 and expressed after induction with galactose and in the presence of substrates that allowed detection of specific desaturase activity. The control strain was *S. cerevisiae* strain 334 containing the unaltered
15 pYES2 vector. The substrates used, the products produced and the indicated desaturase activity were: DGLA (conversion to ARA would indicate $\Delta 5$ -desaturase activity), linoleic acid (conversion to GLA would indicate $\Delta 6$ -desaturase activity; conversion to ALA would indicate $\Delta 15$ -desaturase activity), oleic acid (an endogenous substrate made by *S. cerevisiae*, conversion to
20 linoleic acid would indicate $\Delta 12$ -desaturase activity, which *S. cerevisiae* lacks), or ARA (conversion to EPA would indicate $\Delta 17$ -desaturase activity). The results are provided in Table 1 below. The lipid fractions were extracted as follows: Cultures were grown for 48-52 hours at 15°C. Cells were pelleted by centrifugation, washed once with sterile ddH₂O, and repelleted. Pellets were
25 vortexed with methanol; chloroform was added along with tritridecanoin (as an internal standard). The mixtures were incubated for at least one hour at room temperature or at 4°C overnight. The chloroform layer was extracted and filtered through a Whatman filter with one gram of anhydrous sodium sulfate to remove particulates and residual water. The organic solvents were evaporated
30 at 40°C under a stream of nitrogen. The extracted lipids were then derivatized to fatty acid methyl esters (FAME) for gas chromatography analysis (GC) by

adding 2 ml of 0.5 N potassium hydroxide in methanol to a closed tube. The samples were heated to 95°C to 100°C for 30 minutes and cooled to room temperature. Approximately 2 ml of 14 % boron trifluoride in methanol was added and the heating repeated. After the extracted lipid mixture cooled, 2 ml
5 of water and 1 ml of hexane were added to extract the FAME for analysis by GC. The percent conversion was calculated by dividing the product produced by the sum of (the product produced and the substrate added) and then multiplying by 100. To calculate the oleic acid percent conversion, as no
10 substrate was added, the total linoleic acid produced was divided by the sum of (oleic acid and linoleic acid produced), then multiplying by 100.

Table 1***M. alpina* Desaturase Expression in Baker's Yeast**

CLONE	TYPE OF ENZYME ACTIVITY	% CONVERSION OF SUBSTRATE
pCGR-2	$\Delta 6$	0 (18:2 to 18:3 ω 6)
(canola $\Delta 15$	$\Delta 15$	16.3 (18:2 to 18:3 ω 3)
desaturase)	$\Delta 5$	2.0 (20:3 to 20:4 ω 6)
	$\Delta 17$	2.8 (20:4 to 20:5 ω 3)
	$\Delta 12$	1.8 (18:1 to 18:2 ω 6)
pCGR-4	$\Delta 6$	0
(M. alpina	$\Delta 15$	0
$\Delta 6$ -like, Ma29)	$\Delta 5$	15.3
	$\Delta 17$	0.3
	$\Delta 12$	3.3
pCGR-7	$\Delta 6$	0
(M. alpina	$\Delta 15$	3.8
$\Delta 12$ -like, Ma648	$\Delta 5$	2.2
	$\Delta 17$	0
	$\Delta 12$	63.4

The $\Delta 15$ -desaturase control clone exhibited 16.3% conversion of the substrate. The pCGR-4 clone expressing the Ma29 cDNA converted 15.3% of the 20:3 substrate to 20:4 ω 6, indicating that the gene encodes a $\Delta 5$ -desaturase. The background (non-specific conversion of substrate) was between 0-3% in these cases. The pCGR-5 clone expressing the Ma524 cDNA showed 6% conversion of the substrate to GLA, indicating that the gene encodes a $\Delta 6$ -desaturase. The pCGR-7 clone expressing the Ma648 cDNA converted 63.4% conversion of the substrate to LA, indicating that the gene encodes a $\Delta 12$ -desaturase. Substrate inhibition of activity was observed by using different concentrations of the substrate. When substrate was added to 100 μ M, the percent conversion to product dropped as compared to when substrate was added to 25 μ M (see below). These data show that desaturases with different

substrate specificities can be expressed in a heterologous system and used to produce PUFAs.

Table 2 represents fatty acids of interest as a percent of the total lipid extracted from the yeast host *S. cerevisiae* 334 with the indicated plasmid. No
5 glucose was present in the growth media. Affinity gas chromatography was used to separate the respective lipids. GC/MS was employed to verify the identity of the product(s). The expected product for the *B. napus* $\Delta 15$ -desaturase, α -linolenic acid, was detected when its substrate, linoleic acid, was added exogenously to the induced yeast culture. This finding demonstrates that
10 yeast expression of a desaturase gene can produce functional enzyme and detectable amounts of product under the current growth conditions. Both exogenously added substrates were taken up by yeast, although slightly less of the longer chain PUFA, dihomogamma-linolenic acid (20:3), was incorporated into yeast than linoleic acid (18:2) when either was added in free form to the induced
15 yeast cultures. gamma-linolenic acid was detected when linoleic acid was present during induction and expression of *S. cerevisiae* 334 (pCGR-5). The presence of this PUFA demonstrates $\Delta 6$ -desaturase activity from pCGR-5 (MA524). Linoleic acid, identified in the extracted lipids from expression of *S. cerevisiae* 334 (pCGR-7), classifies the cDNA MA648 from *M. alpina* as the $\Delta 12$ -
20 desaturase.

Table 2
Fatty Acid as a Percentage of Total Lipid Extracted from Yeast

Plasmid in Yeast (enzyme)	18:2 Incorporated	α -18:3 Produced	γ -18:3 Produced	20:3 Incorporated	20:4 Produced	18:1* Present	18:2 Produced
pYES2 (control)	66.9	0	0	58.4	0	4	0
pCGR-2 (Δ 15)	60.1	5.7	0	50.4	0	0.7	0
pCGR-4 (Δ 5)	67	0	0	32.3	5.8	0.8	0
pCGR-5 (Δ 6)	62.4	0	4.0	49.9	0	2.4	0
pCGR-7 (Δ 12)	65.6	0	0	45.7	0	7.1	12.2

100 μ M substrate added

* 18:1 is an endogenous fatty acid in yeast

5 Key To Tables

18:1 =oleic acid
 18:2 =linoleic acid
 α -18:3 = α -linolenic acid
 γ -18:3 = γ -linolenic acid
 18:4 =stearidonic acid
 20:3 =dihomo- γ -linolenic acid
 20:4 =arachidonic acid

Example 7

Expression of $\Delta 5$ Desaturase in Plants

Expression in Leaves

This experiment was designed to determine whether leaves expressing
5 Ma29 (as determined by Northern) were able to convert exogenously applied
DGLA (20:3) to ARA (20:4).

The Ma29 desaturase cDNA was modified by PCR to introduce
convenient restriction sites for cloning. The desaturase coding region has been
inserted into a d35 cassette under the control of the double 35S promoter for
10 expression in *Brassica* leaves (pCGN5525) following standard protocols (*see*
USPN 5,424,200 and USPN 5,106,739). Transgenic *Brassica* plants containing
pCGN5525 were generated following standard protocols (*see* USPN 5,188,958
and USPN 5,463,174).

In the first experiment, three plants were used: a control, LPO04-1, and
15 two transgenics,, 5525-23 and 5525-29. LP004 is a low-linolenic *Brassica*
variety. Leaves of each were selected for one of three treatments: water, GLA
or DGLA. GLA and DGLA were purchased as sodium salts from NuChek Prep
and dissolved in water at 1 mg/ml. Aliquots were capped under N₂ and stored at
-70 degrees C. Leaves were treated by applying a 50 μ l drop to the upper
20 surface and gently spreading with a gloved finger to cover the entire surface.
Applications were made approximately 30 minutes before the end of the light
cycle to minimize any photo-oxidation of the applied fatty acids. After 6 days
of treatment one leaf from each treatment was harvested and cut in half through
the mid rib. One half was washed with water to attempt to remove
25 unincorporated fatty acid. Leaf samples were lyophilized overnight, and fatty
acid composition determined by gas chromatography (GC). The results are
shown in Table 3.

Table 3
Fatty Acid Analysis of Leaves from Ma29 Transgenic *Brassica* Plants

Treatment	SPL	16:00	16:01	18:00	18:01	18:10	18:1v	18:02	18:3g	18:03	18:04	20:00	20:01
	#	%	%	%	%	%	%	%	%	%	%	%	%
Water	33	12.95	0.08	2.63	2.51	1.54	0.98	16.76	0	45.52	0	0.09	0
	34	13.00	0.09	2.67	2.56	1.55	1.00	16.86	0	44.59	0	0.15	0
	35	14.13	0.09	2.37	2.15	1.27	0.87	16.71	0	49.91	0	0.05	0.01
	36	13.92	0.08	2.32	2.07	1.21	0.86	16.16	0	50.25	0	0.05	0
GLA	37	13.79	0.11	2.10	2.12	1.26	0.86	15.90	0.08	46.29	0	0.54	0.01
	38	12.80	0.09	1.94	2.08	1.35	0.73	14.54	0.11	45.61	0	0.49	0.01
	39	12.10	0.09	2.37	2.10	1.29	0.82	14.85	1.63	43.66	0	0.53	0
	40	12.78	0.10	2.34	2.22	1.36	0.86	15.29	1.72	47.22	0	0.50	0.02
DGLA	41	13.71	0.07	2.68	2.16	1.34	0.82	15.92	2.12	46.55	0	0.09	0
	42	14.10	0.07	2.75	2.35	1.51	0.84	16.66	1.56	46.41	0	0.09	0.01
	43	13.62	0.09	2.22	1.94	1.21	0.73	14.68	2.42	46.69	0	0.51	0.01
	44	13.92	0.09	2.20	2.17	1.32	0.85	15.22	2.30	46.05	0	0.53	0.02
	45	12.45	0.14	2.30	2.28	1.37	0.91	15.65	0.07	44.62	0	0.12	0.01
	46	12.67	0.15	2.69	2.50	1.58	0.92	15.96	0.09	42.77	0	0.56	0.01
	47	12.56	0.23	3.40	1.98	1.13	0.86	13.57	0.03	45.52	0	0.51	0.01
	48	13.07	0.24	3.60	2.51	1.63	0.88	13.54	0.04	45.13	0	0.50	0.01
	49	13.26	0.07	2.81	2.34	1.67	0.67	16.04	0.04	43.89	0	0.59	0
	50	13.53	0.07	2.84	2.41	1.70	0.70	16.07	0.02	44.90	0	0.60	0.01

Table 3 - Continued
Fatty Acid Analysis of Leaves from Ma29 Transgenic Brassica Plants

Treatment	SPL	20:02	20:03	20:04	20:05	22:00	22:01	22:02	22:03	22:06	24:0	24:1
	#	%	%	%	%	%	%	%	%	%	%	%
Water	33	0	0	0.29	0	0.01	0.09	16.26	0	0	0.38	0.18
	34	0.01	0	0.26	0	0.14	0.10	16.82	0.02	0.05	0.36	0.27
	35	0.01	0	0.25	0	0.12	0.06	11.29	0.04	0.05	0.29	0.25
	36	0	0.01	0.26	0	0.07	0.04	11.82	0.03	0.36	0.28	0.21
	37	0.02	0	0.21	0	0.18	0.08	15.87	0.06	0.20	0.30	0.17
	38	0.01	0	0.24	0	0.15	0.07	13.64	0.09	0.08	5.89	0.23
GLA	39	0.02	0.01	0.27	0	0.10	0.08	16.25	3.42	0.19	0.37	0.17
	40	0.01	0	0.27	0	0.10	0.10	14.74	0.05	0.10	0.36	0.14
	41	0	0	0.27	0	0.20	0.10	13.15	0.13	0.29	0.33	0.20
	42	0	0	0.28	0	0.11	0.11	12.60	0.02	0.24	0.38	0.13
	43	0.01	0	0.28	0	0.10	0.03	14.73	0.01	0.24	0.34	0.14
	44	0.02	0	0.26	0	0.13	0.07	14.43	0.05	0.16	0.33	0.17
DGLA	45	0.06	1.21	0.26	0	0.07	0.07	18.67	0.02	0.21	0.36	0.13
	46	0	1.94	0.27	0	0.11	0.09	17.97	0.09	0.39	0.41	0.11
	47	0.01	0.69	0.96	0	0.11	0.07	17.96	0	0.22	0.49	0.20
	48	0.01	0.70	0.74	0	0.14	0.09	17.14	0.05	0.32	0.52	0.10
	49	0	0.35	1.11	0	0.10	0.07	17.26	0.07	0.23	0.39	0.18
	50	0	0.20	0.87	0	0.21	0.07	15.73	0.04	0.15	0.37	0.18

Leaves treated with GLA contained from 1.56 to 2.4 wt% GLA. The fatty acid analysis showed that the lipid composition of control and transgenic leaves was essentially the same. Leaves of control plants treated with DGLA contained 1.2-1.9 w% DGLA and background amounts of ARA (.26-.27 wt%).

- 5 Transgenic leaves contained only .2-.7 wt% DGLA, but levels of ARA were increased (.74-1.1 wt%) indicating that the DGLA was converted to ARA in these leaves.

Expression in Seed

- 10 The purpose of this experiment was to determine whether a construct with the seed specific napin promoter would enable expression in seed.

The Ma29 cDNA was modified by PCR to introduce *Xho*I cloning sites upstream and downstream of the start and stop codons, respectively, using the following primers:

Madxho-forward:

- 15 5'-CUACUACUACUACTCGAGCAAGATGGGAACGGACCAAGG
(SEQ ID NO:25)

Madxho-reverse:

5'-CAUCAUCAUCAUCTCGAGCTACTCTTCCTTGGGACGGAG
(SEQ ID NO:26).

- 20 The PCR product was subcloned into pAMP1 (GIBCOBRL) using the CloneAmp system (GIBCOBRL) to create pCGN5522 and the $\Delta 5$ desaturase sequence was verified by sequencing of both strands.

- 25 For seed-specific expression, the Ma29 coding region was cut out of pCGN5522 as an *Xho*I fragment and inserted into the *Sal*I site of the napin expression cassette, pCGN3223, to create pCGN5528. The *Hind*III fragment of pCGN5528 containing the napin 5' regulatory region, the Ma29 coding region, and the napin 3' regulatory region was inserted into the *Hind*III site of pCGN1557 to create pCGN5531. Two copies of the napin transcriptional unit were inserted in tandem. This tandem construct can permit higher expression of

the desaturases per genetic loci. pCGN5531 was introduced into *Brassica napus* cv.LP004 via Agrobacterium mediated transformation.

The fatty acid composition of twenty-seed pools of mature T2 seeds was analyzed by GC. Table 4 shows the results obtained with independent
5 transformed lines as compared to non-transformed LP004 seed. The transgenic seeds containing pCGN5531 contain two fatty acids that are not present in the control seeds, tentatively identified as taxoleic acid (5,9-18:2) and pinolenic acid (5,9,12-18:3), based on their elution relative to oleic and linoleic acid. These would be the expected products of $\Delta 5$ desaturation of oleic and linoleic
10 acids. No other differences in fatty acid composition were observed in the transgenic seeds.

Table 4
Composition of T2 Pooled Seed

	16:0	16:1	18:0	18:1	(5:9)18:2	18:2	(5,9,12)18:3	18:3	20:0	20:1	20:2	22:0	22:1	24:0
	%	%	%	%	%	%	%	%	%	%	%	%	%	%
LP004 control	3.86	0.15	3.05	69.1	0	18.51	0.01	1.65	1.09	1.40	0.03	0.63	0.05	0.42
5531-1	4.26	0.15	3.23	62.33	4.07	21.44	0.33	1.38	0.91	1.04	0.05	0.41	0.03	0.27
5531-2	3.78	0.14	3.37	66.18	4.57	17.31	0.27	1.30	1.03	1.18	0	0.47	0.01	0.30
5531-6	3.78	0.13	3.47	63.61	6.21	17.97	0.38	1.34	1.04	1.14	0.05	0.49	0.02	0.26
5531-10	3.96	0.17	3.28	63.82	5.41	18.58	0.32	1.43	0.98	1.11	0.02	0.50	0	0.31
5531-16	3.91	0.17	3.33	64.31	5.03	18.98	0.33	1.39	0.96	1.11	0	0.44	0	0
5531-28	3.81	0.13	2.58	62.64	5.36	20.95	0.45	1.39	0.83	1.15	0.01	0.36	0.05	0.21

Northern analysis is performed on plants to identify those expressing Ma29. Developing embryos are isolated approximately 25 days post anthesis or when the napin promoter is induced, and floated in a solution containing GLA or DGLA as described in Example 7. Fatty acid analysis of the embryos is then performed by GC to determine the amount of conversion of DGLA to ARA, following the protocol adapted for leaves in Example 7. The amount of ARA incorporated into triglycerides by endogenous *Brassica* acyltransferases is then evaluated by GC analysis as in Example 7.

Example 8

Expression of *M. alpina* $\Delta 6$ Desaturase in *Brassica napus*

The Ma524 cDNA was modified by PCR to introduce cloning sites using the following primers:

Ma524PCR-1 (SEQ ID NO:27)

5'-CUACUACUACUATCTAGACTCGAGACCATGGCTGCTGCT
CCAGTGTG

Ma524PCR-2 (SEQ ID NO:28)

5'-CAUCAUCAUAGGCCTCGAGTTACTGCGCCTTACCCAT

These primers allowed the amplification of the entire coding region and added *Xba*I and *Xho*I sites to the 5'-end and *Xho*I and *Stu*I sites to the 3' end. The PCR product was subcloned into pAMP1 (GIBCOBRL) using the CloneAmp system (GIBCOBRL) to create pCGN5535 and the $\Delta 6$ desaturase sequence was verified by sequencing of both strands.

For seed-specific expression, the Ma524 coding region was cut out of pCGN5535 as an *Xho*I fragment and inserted into the *Sa*I site of the napin expression cassette, pCGN3223, to create pCGN5536. The *Not*I fragment of pCGN5536 containing the napin 5' regulatory region, the Ma524 coding region, and the napin 3' regulatory region was inserted into the *Not*I site of pCGN1557

to create pCGN5538. pCGN5538 was introduced into *Brassica napus* cv.LP004 via Agrobacterium mediated transformation.

5 Maturing T2 seeds were collected from 6 independent transformation events in the greenhouse. The fatty acid composition of single seeds was analyzed by GC. Table 5 shows the results of control LP004 seeds and six 5538 lines. All of the 5538 lines except #8 produced seeds containing GLA. Presence of GLA segregated in these seeds as is expected for the T2 selfed seed population. In addition to GLA, the *M. alpina* $\Delta 6$ desaturase is capable of producing 18:4 (stearidonic) and another fatty acid believed to be the 6,9-18:2.

10 The above results show that desaturases with three different substrate specificities can be expressed in a heterologous system and used to produce poly-unsaturated long chain fatty acids. Exemplified were the production of ARA (20:4) from the precursor 20:3 (DGLA), the production of GLA (18:3) from 18:2 substrate, and the conversion of 18:1 substrate to 18:2, which is the precursor for GLA.

15

Table 5
Fatty Acid Analysis of Seeds from Ma524 Transgenic *Brassica* Plants

SPL #	16:0	16:1	18:0	18:1	6,9	18:2	18:2	18:3ga	18:3	18:4	20:1	22:0	22:1	24:0	24:1
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
LPO04-1	4.33	0.21	3.78	72.49	0	13.97	0	1.7	0	1.34	0.71	0.02	0.58	0.27	
-2	4.01	0.16	3.09	73.59	0	14.36	0.01	1.4	0	1.43	0.66	0.02	0.5	0.2	
-3	4.12	0.19	3.56	70.25	0	17.28	0	1.57	0	1.28	0.5	0.02	0.39	0.2	
-4	4.22	0.2	2.7	70.25	0	17.86	0	1.61	0	1.31	0.53	0.02	0.4	0.24	
-5	4.02	0.16	3.41	72.91	0	14.45	0.01	1.45	0	1.37	0.7	0.02	0.51	0.26	
-6	4.22	0.18	3.23	71.47	0	15.92	0.01	1.52	0	1.32	0.69	0.02	0.51	0.27	
-7	4.1	0.16	3.47	72.06	0	15.23	0	1.52	0	1.32	0.63	0.03	0.49	0.23	
-9	4.01	0.17	3.71	72.98	0	13.97	0.01	1.41	0	1.45	0.74	0.03	0.58	0.23	
-10	4.04	0.16	3.57	70.03	0	17.46	0	1.5	0	1.33	0.61	0.03	0.36	0.24	
5538-1-1	4.61	0.2	3.48	68.12	1.37	10.68	7.48	1.04	0.33	1.19	0.49	0.02	0.33	0.13	
-2	4.61	0.22	3.46	68.84	1.36	10.28	7.04	1.01	0.31	1.15	0.48	0.02	0.39	0	
-3	4.78	0.24	3.24	65.86	0	21.36	0	1.49	0	1.08	0.46	0.02	0.38	0.22	
-4	4.84	0.3	3.89	67.64	1.67	9.9	6.97	1.02	0.36	1.14	0.53	0.02	0.5	0.18	
-5	4.64	0.2	3.58	64.5	3.61	8.85	10.14	0.95	0.48	1.19	0.47	0.01	0.33	0.12	
-6	4.91	0.27	3.44	66.51	1.48	11.14	7.74	1.15	0.33	1.08	0.49	0.02	0.34	0.13	
-7	4.87	0.22	3.24	65.78	1.27	11.92	8.38	1.2	0	1.12	0.47	0.02	0.37	0.16	

Table 5
Fatty Acid Analysis of Seeds from Ma524 Transgenic *Brassica* Plants

SPL #	16:0	16:1	18:0	18:1	6,9	18:2	18:2	18:3ga	18:3	18:4	20:1	22:0	22:1	24:0	24:1
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
-8	4.59	0.22	3.4	70.77	0	16.71	0	1.35	0	1.14	0.48	0.02	0.39	0.15	
-9	4.63	0.23	3.51	69.66	2.01	8.77	7.24	0.97	0	1.18	0.52	0.02	0.3	0.11	
-10	4.56	0.19	3.55	70.68	0	16.89	0	1.37	0	1.22	0.54	0.02	0.22	0.03	
5538-3-1	4.74	0.21	3.43	67.52	1.29	10.91	7.77	1.03	0.28	1.11	0.5	0.02	0.35	0.14	
-2	4.72	0.21	3.24	67.42	1.63	10.37	8.4	0.99	0	1.12	0.49	0.02	0.36	0.15	
-3	4.24	0.21	3.52	71.31	0	16.53	0	1.33	0	1.12	0.45	0.02	0.4	0.14	
-4	4.64	0.21	3.45	67.92	1.65	9.91	7.97	0.91	0.33	1.14	0.47	0.02	0.37	0.14	
-5	4.91	0.25	3.31	67.19	0	19.92	0.01	1.39	0	1.05	0.48	0.02	0.37	0.14	
-6	4.67	0.21	3.25	67.07	1.23	11.32	8.35	0.99	0	1.16	0.47	0.02	0.33	0.16	
-7	4.53	0.19	2.94	64.8	4.94	8.45	9.95	0.93	0.44	1.13	0.37	0.01	0.27	0.12	
-8	4.66	0.22	3.68	67.33	0.71	12	6.99	1.1	0.24	1.18	0.48	0.03	0.36	0.17	
-9	4.65	0.24	3.11	67.42	0.64	12.71	6.93	1.16	0.25	1.08	0.45	0.02	0.32	0.17	
-10	4.88	0.27	3.33	65.75	0.86	12.89	7.7	1.1	0.24	1.08	0.46	0.01	0.34	0.16	
5538-4-1	4.65	0.24	3.8	62.41	0	24.68	0	1.6	0.01	0.99	0.45	0.02	0.33	0.13	
-2	5.37	0.31	3	57.98	0.38	18.04	10.5	1.41	0	0.99	0.48	0.02	0.3	0.19	
-3	4.61	0.22	3.07	63.62	0.3	16.46	7.67	1.2	0	1.18	0.45	0.02	0.29	0.14	

Table 5
Fatty Acid Analysis of Seeds from Ma524 Transgenic *Brassica* Plants

SPL #	16:0	16:1	18:0	18:1	6,9	18:2	18:2	18:3ga	18:3	18:4	20:1	22:0	22:1	24:0	24:1
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
-4	4.39	0.19	2.93	65.97	0	22.36	0	1.45	0	1.17	0.41	0.03	0.32	0.15	
-5	5.22	0.29	3.85	62.1	2.35	10.25	11.39	0.93	0.41	1.04	0.6	0.02	0.47	0.17	
-6	4.66	0.18	2.85	66.79	0.5	13.03	7.66	0.97	0.22	1.28	0.42	0.02	0.31	0.14	
-7	4.85	0.26	3.03	57.43	0.26	28.04	0.01	2.59	0.01	1.13	0.56	0.02	0.4	0.23	
-8	5.43	0.28	2.94	54.8	1.84	13.79	15.67	1.36	0.53	1.1	0.55	0.02	0.35	0.19	
-9	4.88	0.24	3.32	62.3	0.58	14.86	9.04	1.34	0.29	1.13	0.52	0.02	0.37	0.19	
-10	4.53	0.2	2.73	64.2	0.07	24.15	0	1.52	0	1.09	0.39	0.02	0.27	0.17	
5538-5-1	4.5	0.15	3.35	66.71	0.88	11.7	8.38	1.04	0.3	1.24	0.49	0.02	0.29	0.17	
-2	4.77	0.23	3.06	62.67	0.68	15.2	8.8	1.31	0.28	1.15	0.46	0.02	0.3	0.19	
-3	4.59	0.22	3.61	64.35	2.29	9.95	10.57	1.01	0.45	1.21	0.48	0.02	0.26	0.16	
-4	4.86	0.26	3.4	67.69	0.65	12.24	6.61	1.09	0.23	1.07	0.45	0.02	0.32	0.14	
-5	4.49	0.21	3.3	69.25	0.04	16.51	2.18	1.2	0	1.11	0.44	0.02	0.33	0.16	
-6	4.5	0.21	3.47	70.48	0.08	14.9	2.19	1.22	0	1.13	0.49	0.02	0.33	0.16	
-7	4.39	0.21	3.44	67.59	2.38	9.24	8.98	0.89	0	1.18	0.44	0.02	0.28	0.14	
-8	4.52	0.22	3.17	68.33	0.01	18.91	0.73	1.32	0.01	1.08	0.45	0.02	0.29	0.17	
-9	4.68	0.2	3.05	64.03	1.93	11.03	11.41	1.02	0.01	1.15	0.39	0.02	0.21	0.15	

Table 5
Fatty Acid Analysis of Seeds from Ma524 Transgenic *Brassica* Plants

SPL #	16:0	16:1	18:0	18:1	6,9	18:2	18:2	18:3ga	18:3	18:4	20:1	22:0	22:1	24:0	24:1
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
-10	4.57	0.2	3.1	67.21	0.61	12.62	7.68	1.07	0.25	1.14	0.43	0.02	0.25	0.15	
5538-8-1	4.95	0.26	3.14	64.04	0	23.38	0	1.54	0	0.99	0.42	0.02	0.38	0.17	
-2	4.91	0.26	3.71	62.33	0	23.97	0	1.77	0	0.95	0.53	0.02	0.42	0.19	
-3	4.73	0.25	4.04	63.83	0	22.36	0.01	1.73	0	1.05	0.55	0.02	0.45	0.16	
-4	5.1	0.35	3.8	60.45	0	24.45	0.01	2.13	0	1.07	0.65	0.03	0.53	0.24	
-5	4.98	0.3	3.91	62.48	0	23.44	0	1.77	0	1.01	0.51	0.01	0.43	0.21	
-6	4.62	0.21	3.99	66.14	0	20.38	0	1.48	0	1.15	0.53	0.02	0.48	0.19	
-7	4.64	0.22	3.55	64.6	0	22.65	0	1.38	0	1.09	0.45	0.02	0.41	0.19	
-8	5.65	0.38	3.18	56.6	0	30.83	0.02	0.02	0	0.98	0.55	0.03	0.39	0.26	
-9	8.53	0.63	6.9	51.76	0	26.01	0	0.01	0	1.41	1.21	0.07	0.96	0.33	
-10	5.52	0.4	3.97	57.92	0	28.95	0	0.02	0	0.95	0.52	0.02	0.41	0.16	
5538-10-1	4.44	0.19	3.5	68.42	0	19.51	0	1.32	0	1.14	0.45	0.02	0.31	0.16	
-2	4.57	0.21	3.07	66.08	0	21.99	0.01	1.36	0	1.12	0.41	0.02	0.31	0.16	
-3	4.63	0.21	3.48	67.43	0	20.27	0.01	1.32	0	1.12	0.46	0.02	0.21	0.08	
-4	4.69	0.19	3.22	64.62	0	23.16	0	1.35	0	1.08	0.46	0.02	0.33	0.2	
-5	4.58	0.2	3.4	68.75	0	20.17	0.01	0.02	0	1.1	0.45	0.02	0.34	0.17	

Table 5
Fatty Acid Analysis of Seeds from Ma524 Transgenic *Brassica* Plants

SPL #	16:0	16:1	18:0	18:1	6,9	18:2	18:2	18:3ga	18:3	18:4	20:1	22:0	22:1	24:0	24:1
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
-8	4.55	0.21	0	73.55	0.05	14.91	2.76	1.21	0.07	1.24	0.51	0.02	0.19	0	
-9	4.58	0.21	3.28	66.19	0	21.55	0	1.35	0	1.12	0.43	0.02	0.33	0.16	
-10	4.52	0.2	3.4	68.37	0	19.33	0.01	1.3	0	1.13	0.46	0.02	0.35	0.18	

Example 9

Expression of *M. alpina* $\Delta 12$ desaturase in *Brassica napus*

The Ma648 cDNA was modified by PCR to introduce cloning sites using the following primers:

5 Ma648PCR-for (SEQ ID NO:29)
 5'-CUACUACUACUAGGATCCATGGCACCTCCCAACACT
 Ma648PCR-rev (SEQ ID NO:30)
 5'-CAUCAUCAUCAUGGTACCTCGAGTTACTTCTTGAAAAAGAC

10 These primers allowed the amplification of the entire coding region and added a BamHI site to the 5' end and KpnI and XhoI sites to the 3' end. The PCR product was subcloned into pAMP1 (GIBCOBRL) using the CloneAmp system (GIBCOBRL) to create pCGN5540 and the $\Delta 12$ desaturase sequence was verified by sequencing of both strands.

15 For seed-specific expression, the Ma648 coding region was cut out of pCGN5540 as a BamHI/XhoI fragment and inserted between the BglII and XhoI sites of the napin expression cassette, pCGN3223, to create pCGN5542. The Asp718 fragment of pCGN5541 containing the napin 5' regulatory region, the Ma648 coding region, and the napin 3' regulatory region was inserted into the Asp718 site of pCGN5138 to create pCGN5542. pCGN5542 was
20 introduced into two varieties of *Brassica napus* via *Agrobacterium* mediated transformation. The commercial canola variety, SP30021, and a low-linolenic line, LP30108 were used.

25 Mature selfed T2 seeds were collected from 19 independent LP30108 transformation events and a non-transformed control grown in the greenhouse. These seeds are expected to be segregating for the $\Delta 12$ desaturase transgene. The fatty acid composition of 20-seed pools was analyzed by GC. The results are shown in Table 6. All transformed lines contained increased levels of 18:2, the product of the $\Delta 12$ desaturase. Levels of 18:3 were not significantly increased in these plants. Events # 11 and 16 showed the greatest accumulation

of 18:2 in the pooled seeds. To investigate the segregation of 18:2 levels in the T2 seeds and to identify individual plants to be taken on to subsequent generations, half-seed analysis was done. Seeds were germinated overnight in the dark at 30 degrees on water-soaked filter paper. The outer cotyledon was excised for GC analysis and the rest of the seedling was planted in soil. Results of some of these analyses are shown in Table 7. Individual T2 seeds containing the *M. alpina* $\Delta 12$ desaturase accumulated up to 60% 18:2 in the seeds. Sample 97xx1116 #59 is an example of a null segregant. Even in the highest 18:2 accumulators, levels of 18:3 were increased only slightly. These and other individually selected T2 plants were grown in the greenhouse and in the field to produce T3 seed.

Mature selfed T2 seeds were collected from 20 independent SP30021 transformation events and a non-transformed control grown in the greenhouse. These seeds are expected to be segregating for the $\Delta 12$ desaturase transgene. The fatty acid composition of 20-seed pools was analyzed by GC. The data are presented in Table 8. All transformed lines contained increased levels of 18:2, the product of the $\Delta 12$ desaturase. As in the low-linolenic LP30108 line, levels of 18:3 were not significantly increased. Events # 4 and 12 showed the greatest accumulation of 18:2 in the pooled seeds. To investigate the segregation of 18:2 levels in the T2 seeds and to identify individual plants to be taken on to subsequent generations, half-seed analysis was done. Seeds were germinated overnight in the dark at 30 degrees on water-soaked filter paper. The outer cotyledon was excised for GC analysis and the rest of the seedling was planted in soil. Results of some of these analyses are shown in Table 9. Samples 97xx1157 #88 and #18 are examples of null segregants for 5542-SP30021-4 and 5542-SP30021-12 respectively. These and other individually selected T2 plants were grown in the greenhouse and in the field to produce T3 seed

Table 6

CYCLE ID	SPL NO	STRAIN ID	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0
97XX1098	45	5542-LP30108-16	7.04	0.43	1.12	18.01	66.36	4.76	0.5	0.84	0.3	0.44
97XX1098	22	5542-LP30108-16	5.17	0.29	2.11	22.01	65.18	3.15	0.63	0.75	0.21	0.36
97XX1098	40	5542-LP30108-16	4.99	0.2	2.05	23.91	63.13	3.3	0.73	0.85	0.23	0.49
97XX1098	28	5542-LP30108-16	4.47	0.19	1.75	26.7	62.39	2.46	0.58	0.85	0.2	0.32
97XX1098	2	5542-LP30108-16	4.54	0.21	1.66	26.83	61.89	2.9	0.55	0.82	0.18	0.33
97XX1098	58	5542-LP30108-16	6.05	0.31	1.36	24.11	61.36	3.8	0.72	1.13	0.26	0.58
97XX1098	83	5542-LP30108-16	5.13	0.17	2.03	27.05	60.93	2.62	0.7	0.71	0.14	0.4
97XX1098	34	5542-LP30108-16	4.12	0.19	1.44	29.35	60.54	2.53	0.43	0.89	0.17	0.25
97XX1116	37	5542-LP30108-11	4	0.14	2.43	23.29	63.99	2.6	0.58	0.69	0.71	1.11
97XX1116	88	5542-LP30108-11	3.8	0.18	2.04	23.59	63.93	2.95	0.54	0.81	0.99	0.82
97XX1116	36	5542-LP30108-11	4.15	0.2	1.51	25.94	62.14	2.74	0.47	0.87	0.79	0.81
97XX1116	31	5542-LP30108-11	6.29	0.35	1.04	24.14	60.91	4.02	0.55	0.91	0.75	0.72
97XX1116	10	5542-LP30108-11	6.97	0.4	3.36	18.9	60.66	4.68	1.2	0.7	0.53	1.71
97XX1116	32	5542-LP30108-11	3.96	0.16	2.61	26.73	60.54	3.38	0.66	0.87	0.2	0.62
97XX1116	55	5542-LP30108-11	4.26	0.22	0.98	28.57	59.94	3.24	0.4	0.68	0.71	0.75
97XX1116	12	5542-LP30108-11	4.17	0.23	1.42	28.61	59.52	3.26	0.51	0.95	0.29	0.67

Table 6

CYCLE ID	SPL NO	STRAIN ID	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0
97XX1116	86	5542-LP30108-11	4.23	0.3	1.09	28.34	59.2	3.95	0.48	0.91	0.55	0.71
97XX1116	61	5542-LP30108-11	4.13	0.16	1.92	30.18	58.67	2.65	0.56	0.88	0.25	0.41
97XX1116	60	5542-LP30108-11	4.42	0.26	1.61	28.77	58.6	3.26	0.53	0.85	0.68	0.75
97XX1116	91	5542-LP30108-11	7.82	0.67	2.37	17.97	58.43	4.85	0.94	0.86	3.87	1.71
97xx1116	59	5542-LP30108-11	3.56	0.2	1.6	65.5	23.03	2.23	0.52	1.54	0.49	0.69

Table 7

	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0
	%	%	%	%	%	%	%	%	%	%
5542-LP30108-1	4.6	0.15	1.93	50.44	38.54	2.06	0.65	1.11	0.09	0.37
5542-LP30108-2	4.63	0.17	1.78	41.11	47.53	2.46	0.62	1.02	0.14	0.38
5542-LP30108-3	4.96	0.18	2.07	48.16	40.01	2.17	0.73	1.13	0.1	0.39
5542-LP30108-4	4.36	0.15	1.94	46.51	42.57	1.95	0.64	1.06	0.11	0.35
5542-LP30108-5	4.45	0.14	2.19	49.54	39.13	2.14	0.72	1.14	0.11	0.38
5542-LP30108-6	4.97	0.16	1.86	49.23	39.2	2.17	0.7	1.12	0.11	0.41
5542-LP30108-7	4.46	0.13	2.72	39.6	48.65	2.02	0.81	0.96	0.13	0.4
5542-LP30108-8	4.63	0.18	1.78	47.86	41	2.31	0.62	1.09	0.11	0.36
5542-LP30108-9	4.64	0.16	1.75	42.5	46.57	2.2	0.61	1	0.13	0.35
5542-LP30108-10	4.46	0.15	2.37	43.61	45.29	1.77	0.71	1.02	0.12	0.36
5542-LP30108-11	4.58	0.25	1.88	37.08	50.95	2.94	0.64	0.96	0.16	0.42
5542-LP30108-12	4.46	0.18	1.69	43.62	45.36	2.44	0.59	1.09	0.14	0.34
5542-LP30108-13	4.45	0.15	2.33	51	37.71	1.91	0.75	1.12	0.09	0.4
5542-LP30108-14	4.3	0.16	2.04	45.93	42.78	2.46	0.66	1.07	0.14	0.37
5542-LP30108-15	4.18	0.16	2.17	43.79	45.2	2.14	0.68	1.04	0.15	0.36
5542-LP30108-16	5.04	0.18	1.89	32.32	55.78	2.68	0.63	0.84	0.2	0.36

Table 7

	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0
	%	%	%	%	%	%	%	%	%	%
5542-LP30108-18	4.2	0.14	2.23	50.63	38.51	1.79	0.72	1.15	0.1	0.37
5542-LP30108-19	4.63	0.18	1.81	52.51	36.26	2.12	0.68	1.19	0.1	0.4
5542-LP30108-20	4.77	0.15	2.78	39.76	48.06	2.25	0.75	0.91	0.13	0.36
LP30108 control	4.31	0.22	2.05	66.15	22.59	1.87	0.77	1.3	0.07	0.44

Table 8

STRAIN ID	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0
5542-SP30021-1	4.37	0.17	2.17	40.26	39.43	11.06	0.74	1.14	0.14	0.42
5542-SP30021-2	4.33	0.18	1.51	43.07	36.03	12.57	0.57	1.21	0.14	0.33
5542-SP30021-3	5.2	0.22	3.1	43.7	37.04	8.03	0.92	1.06	0.13	0.48
5542-SP30021-4	4.37	0.15	1.94	34.26	45.12	12.04	0.6	0.96	0.17	0.3
5542-SP30021-5	4.15	0.17	1.73	48.98	31.13	11.41	0.63	1.26	0.13	0.35
5542-SP30021-6	4.52	0.17	1.92	38.1	42.39	10.53	0.67	1.04	0.18	0.39
5542-SP30021-7	4.58	0.18	1.66	41.87	37.52	11.8	0.62	1.14	0.15	0.36
5542-SP30021-8	4.46	0.17	1.59	42.69	36.93	11.88	0.59	1.14	0.14	0.35
5542-SP30021-9	4.63	0.19	1.69	39.89	39.75	11.48	0.62	1.09	0.15	0.38
5542-SP30021-10	4.74	0.16	1.79	39.19	40.51	11.42	0.63	0.99	0.13	0.34
5542-SP30021-11	4.57	0.16	1.71	38.13	42	11.15	0.62	1.04	0.18	0.36
5542-SP30021-12	4.05	0.16	2.04	35.44	43.47	12.45	0.62	1.07	0.21	0.33
5542-SP30021-13	4.37	0.15	1.79	38.74	41.28	11.36	0.62	1.04	0.16	0.35
5542-SP30021-14	4.32	0.16	1.47	42.32	37.17	12.3	0.54	1.16	0.16	0.32
5542-SP30021-15	4.25	0.18	1.65	44.96	34.28	12.39	0.59	1.13	0.14	0.32

Table 8

STRAIN ID	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0
5542-SP30021-16	4.53	0.17	1.91	42.13	38.32	10.51	0.67	1.12	0.14	0.38
5542-SP30021-17	4.16	0.19	1.7	50.65	29.3	11.4	0.61	1.29	0.11	0.36
5542-SP30021-18	4.24	0.17	1.68	44.47	35.46	11.52	0.6	1.19	0.14	0.34
5542-SP30021-19	4.1	0.18	1.8	46.67	33.87	10.86	0.63	1.24	0.13	0.37
5542-SP30021-20	4.3	0.17	1.64	39.6	40.39	11.53	0.57	1.12	0.16	0.32
SP30021	4.38	0.21	1.47	56.51	22.59	12.04	0.62	1.45	0.11	0.39

Table 9

CYCLE ID	SPL NO	STRAIN ID	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0
97XX1156	96	5542-SP30021-4	3.71	0.13	1.36	29.29	51.74	11.57	0.41	0.85	0.18	0.46
97XX1156	50	5542-SP30021-4	2.95	0.11	1.33	28.78	50.97	13.83	0.3	0.99	0.28	0.32
97XX1158	10	5542-SP30021-4	4.05	0.16	2.47	31.18	50.88	8.77	0.67	0.89	0.22	0.33
97XX1158	32	5542-SP30021-4	3.56	0.15	1.44	30.73	50.1	11.86	0.47	0.91	0.21	0.22
97XX1158	56	5542-SP30021-4	4.44	0.19	3.09	30.64	49.71	9.39	0.83	0.79	0.2	0.4
97XX1157	80	5542-SP30021-4	4.05	0.18	1.32	27.41	49.59	14.81	0.53	1.19	0.29	0.4
97XX1158	39	5542-SP30021-4	4.04	0.15	2.98	28.62	49.52	12.28	0.69	0.86	0.31	0.27
97XX1156	17	5542-SP30021-4	3.65	0.15	2.43	29.38	49.42	12.3	0.52	0.92	0.67	0.35
97XX1156	60	5542-SP30021-4	3.75	0.17	1.7	30.03	49.13	12.87	0.51	1.01	0.27	0.35
97XX1157	83	5542-SP30021-4	4.15	0.2	1.77	29.72	49.08	12.22	0.66	1.21	0.16	0.52
97XX1157	86	5542-SP30021-4	3.6	0.14	1.12	27.65	49.01	16.05	0.48	1.21	0.33	0.08
97XX1158	77	5542-SP30021-4	4.14	0.17	1.58	31.98	48.82	10.72	0.65	1	0.28	0.44
97XX1157	88	5542-SP30021-4	3.36	0.15	1.22	56.42	21.63	13.78	0.58	1.85	0.06	0.65

Table 9

CYCLE ID	SPL NO	STRAIN ID	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0
97XX1157	39	5542-SP30021-12	2.84	0.04	1.84	29.6	53.16	9.52	0.57	1.32	0.35	0.48
97XX1157	55	5542-SP30021-12	3.28	0.1	2.18	30.36	52.27	9.26	0.63	1.15	0.22	0.41
97XX1157	10	5542-SP30021-12	3.5	0.06	1.51	29.78	50.98	11.13	0.64	1.45	0.4	0.26
97XX1157	41	5542-SP30021-12	3.31	0.08	1.64	30.18	50.51	11.59	0.57	1.27	0.24	0.41
97XX1157	35	5542-SP30021-12	3.31	0.09	1.57	30.36	50.1	12.17	0.5	1.15	0.23	0.35
97XX1157	1	5542-SP30021-12	3.45	0.11	2.88	32.11	49.45	8.69	0.82	1.22	0.27	0.63
97XX1157	16	5542-SP30021-12	2.91	0.09	1.52	29.35	48.88	14.26	0.58	1.39	0.15	0.3
97XX1157	50	5542-SP30021-12	3.29	0.09	2.13	33.23	48.78	9.87	0.67	1.06	0.18	0.47
97XX1157	25	5542-SP30021-12	2.83	0.05	1.4	33.22	48.52	11.22	0.5	1.33	0.26	0.42
97XX1157	57	5542-SP30021-12	2.94	0.13	1.46	32.85	47.58	12.21	0.57	1.31	0.27	0.47
97XX1157	56	5542-SP30021-12	3.01	0.07	1.63	31.53	47	14.02	0.59	1.31	0.28	0.23
97XX1157	6	5542-SP30021-12	3.9	0.13	1.5	32.43	46.98	12.45	0.52	1.11	0.21	0.49
97XX1157	18	5542-SP30021-12	3.88	0.16	1.73	57.94	22.33	10.51	0.74	1.68	0.11	0.64

Example 10

Simultaneous expression of *M. alpina* $\Delta 6$ and $\Delta 12$ desaturases in *Brassica napus*

5 In order to express the *M. alpina* $\Delta 6$ and $\Delta 12$ desaturases from the same T-DNA, the following construct for seed-specific expression was made.

 The NotI fragment of pCGN5536 containing the napin 5' regulatory region, the Ma524 coding region, and the napin 3' regulatory region was inserted into the NotI site of pCGN5542 to create pCGN5544. The
10 expression modules were oriented in such a way that the direction of transcription from Ma524 and Ma648 and the nptII marker is the same.

 PCGN5544 was introduced into *Brassica napus* cv.LP30108 via *Agrobacterium* mediated transformation. Mature selfed T2 seeds were collected from 16 independent LP30108 transformation events and a non-transformed
15 control that were grown in the greenhouse. These seeds are expected to be segregating for the $\Delta 6 + \Delta 12$ desaturase transgene. The fatty acid composition of 20-seed pools was analyzed by GC. The results are presented in Table 10. All but one of the lines (5544-LP30108-3) shows an altered oil composition as compared to the controls. GLA was produced in all but three of the lines (-3, -4,
20 -11); two of the three without GLA (-4, -11) showed increased 18:2 indicative of expression of the $\Delta 12$ desaturase. As a group, the levels of GLA observed in plants containing the double $\Delta 6 + \Delta 12$ construct (pCGN5544) were higher than those of plants containing pCGN5538 ($\Delta 6$ alone). In addition, levels of the $\Delta^{6,9}$ 18:2 are much reduced in the plants containing the $\Delta 12 + \Delta 6$ as compared to $\Delta 6$
25 alone. Thus, the combination of $\Delta 6$ and $\Delta 12$ desaturases on one T-DNA leads to the accumulation of more GLA and fewer side products than expression of $\Delta 6$ desaturase alone. To investigate the segregation of GLA levels in the T2 seeds and to identify individual plants to be taken on to subsequent generations, half-seed analysis was done. Seeds were germinated overnight in the dark at 30
30 degrees on water-soaked filter paper. The outer cotyledon was excised for GC analysis and the rest of the seedling was planted in soil. Results of some of

these analyses are shown in Table 11. As expected for the T2 population, levels of GLA and 18:2 are segregating in the individual seeds. GLA content of up to 60% of total fatty acids was observed in individual seeds. Individual events were selected to be grown in the greenhouse and field for production of T3 seed.

5

Transgenic plants including *Brassica*, soybean, safflower, corn flax and sunflower expressing the constructs of this invention can be a good source of GLA.

Typical sources of GLA such as borage produce at most 25% GLA. In contrast the plants in Table 10 contain up to 30% GLA. Furthermore, the individual seeds shown in Table 11 contain up to 60% GLA.

10

Table 10

	16:0	16:1	18:0	18:1	18:2	18:2	18:3	18:3	18:4	20:0	20:1	22:0
						$\Delta 6,9$	$\Delta 9,12$	$\Delta 6,9,12$	$\Delta 9,12,15$			
	%	%	%	%	%	%	%	%	%	%	%	%
5544-LP30108-1	4.54	0.17	1.91	49.96	0	30.98	7.97	1.85	0.11	0.68	1.17	0.41
5544-LP30108-2	4.69	0.19	2.15	38.49	0	33.94	16.21	1.73	0.25	0.72	0.96	0.41
5544-LP30108-3	4.26	0.2	1.97	66.68	0	22.13	0.08	1.96	0.01	0.73	1.33	0.42
5544-LP30108-4	4.59	0.24	1.76	44.21	0	44.54	0.02	2.19	0.01	0.62	1.08	0.4
5544-LP30108-5	4.5	0.18	2.28	47.57	0	26.41	14.42	1.71	0.22	0.78	1.1	0.43
5544-LP30108-6	4.51	0.16	2.12	31.95	0.01	26.94	29.8	1.41	0.5	0.81	1.02	0.51
5544-LP30108-7	4.84	0.21	1.68	38.24	0	32.27	18.21	1.87	0.33	0.66	1.04	0.43
5544-LP30108-10	5	0.28	1.86	41.17	0	46.54	0.36	2.58	0.02	0.6	0.91	0.37
5544-LP30108-11	4.57	0.2	1.74	47.29	0	41.49	0.03	2.22	0.01	0.64	1.17	0.4
5544-LP30108-12	4.87	0.18	2.65	34.53	0	30.37	23.12	1.46	0.36	0.83	0.95	0.45
5544-LP30108-13	4.41	0.16	2.32	40.82	0.11	26.8	21.05	1.53	0.37	0.77	1.06	0.42
5544-LP30108-14	4.38	0.2	2.21	29.91	0.16	28.01	30.62	1.46	0.59	0.76	0.97	0.47
5544-LP30108-15	4.79	0.22	2.23	23.42	0.02	28.73	35.68	1.51	0.77	0.87	0.89	0.56
5544-LP30108-16	4.54	0.18	1.78	40.81	0	35.24	12.83	1.95	0.27	0.68	1.02	0.43
5544-LP30108-17	4.63	0.18	2.28	46.96	0	31.06	10.6	1.7	0.14	0.76	1.06	0.42
5544-LP30108-20	4.87	0.29	1.44	31.81	0.15	23.51	32.85	1.64	0.69	0.89	0.96	0.67

Table 10

16:0	16:1	18:0	18:1	18:2	18:2	18:3	18:3	18:4	20:0	20:1	22:0	
				$\Delta 6,9$	$\Delta 9,12$	$\Delta 6,9,12$	$\Delta 9,12,15$					
%	%	%	%	%	%	%	%	%	%	%	%	
LP30108 control	3.89	0.25	1.19	67.73	0	22.46	0.1	1.97	0	0.54	1.32	0.44

Table 11

CYCLE ID	SPL NO	STRAIN ID	16:0	16:1	18:0	18:1	18:2_Δ6,9	18:2_Δ9,12	18:3_Δ6,9, 12	18:3_Δ9,12, 15	18:4	20:0	20:1
97XX1333	64	5544-LP30108-20	6.53	0.15	0.98	23.33	0.01	21.1	43.3	1.34	0.84	0.52	0.97
97XX1333	65	5544-LP30108-20	6.9	0.29	1.17	8.89	0.03	15.07	60.5	1.12	2.23	0.98	0.86
97XX1333	66	5544-LP30108-20	8.15	0.2	3.6	16.87	0.11	16.05	48.23	1.1	1.18	1.71	0.66
97XX1333	67	5544-LP30108-20	8.85	0.35	1.2	14.49	0.01	25.66	43.98	1.8	1.03	0.65	0.76
97XX1333	68	5544-LP30108-20	6.05	0.16	1.27	17.85	0.16	16.13	53.16	1.14	1.25	0.71	0.85
97XX1333	69	5544-LP30108-20	7.16	0.21	1.33	11.51	0.09	17.42	56.13	1.41	1.58	0.93	0.68
97XX1333	70	5544-LP30108-20	3.46	0.04	1.76	18.38	0.03	22.55	48.55	1.22	1.04	0.83	0.95
97XX1333	71	5544-LP30108-20	3.71	0.05	1.74	16.11	0.01	26.93	45.79	1.47	1.02	0.89	1
97XX1333	72	5544-LP30108-20	3.5	0.04	1.76	23.74	0.02	35.38	30.82	1.87	0.58	0.65	0.89
97XX1333	73	5544-LP30108-20	4.67	0.11	1.87	17.98	0.04	22.47	47.89	1.17	0.89	0.93	0.88
97XX1333	74	5544-LP30108-20	4.52	0.09	1.86	13.77	0.03	20.9	52.96	1.31	1.19	1.03	0.88
97XX1333	75	5544-LP30108-20	5.26	0.13	1.64	16.46	0.05	21.75	49.42	1.25	1.08	0.83	0.86
97XX1333	76	5544-LP30108-20	7.61	0.21	1.44	12.49	0.33	17	55.31	1.18	1.59	0.88	0.74
97XX1333	77	5544-LP30108-20	6.42	0.15	1.51	10.79	0.09	15.96	58.77	1.12	1.53	0.98	0.85
97XX1333	78	5544-LP30108-20	4.59	0.16	0.93	12.1	0.08	15.94	60.15	1.12	1.69	0.74	0.88
97XX1333	79	5544-LP30108-20	5.24	0.09	1.94	14.08	0.21	19.79	53.58	1.05	1.03	0.96	0.84

Table 11

CYCLE ID	SPL NO	STRAIN ID	16:0	16:1	18:0	18:1	18:2_Δ6,9	18:2_Δ9,12	18:3_Δ6,9, 12	18:3_Δ9,12, 15	18:4	20:0	20:1
97XX1333	80	5544-LP30108-20	4.38	0.08	1.66	22.25	0	30.79	35.49	2.16	0.72	0.66	0.84
97XX1333	81	5544-LP30108-20	4.05	0.05	1.44	24.16	0.04	24.86	40.89	1.42	0.79	0.63	0.84
97XX1333	82	5544-LP30108-20	3.29	0.05	1.9	19.66	0	23.83	46.48	1.27	0.87	0.78	0.81
97XX1333	83	5544-LP30108-20	4.82	0.08	1.99	17.27	0.1	20.69	49.73	1.22	1.06	0.98	0.82
97XX1333	84	5544-LP30108-20	5.33	0.1	1.77	13.6	0.03	21.44	51.74	1.52	1.21	0.98	0.93
97XX1333	85	5544-LP30108-20	3.3	0.05	1.2	68.23	0	22.09	0.01	2.27	0	0.57	1.57
97XX1333	86	5544-LP30108-20	3.23	0.05	1.54	28.15	0.01	36.4	25.91	1.99	0.43	0.59	0.97
97XX1333	87	5544-LP30108-20	4.38	0.1	1.16	60.94	2.85	8.35	17.61	1.26	0.69	0.54	1.39
97XX1333	88	5544-LP30108-20	4.4	0.09	1.34	38.42	0.02	34.74	16.61	2.12	0.32	0.53	0.82
97XX1278	16	5544-LP30108-15	3.62	0.11	1.22	27.23	0	30.9	32.87	1.41	0.48	0.46	0.97
97XX1278	17	5544-LP30108-15	3.68	0.13	1.26	45.29	0	44.79	0.72	1.77	0.01	0.43	1.24
97XX1278	18	5544-LP30108-15	4.08	0.15	1.49	22.34	0	28.37	39.37	1.22	0.64	0.55	0.88
97XX1278	19	5544-LP30108-15	3.51	0.1	1.01	35.44	0	44.12	11.7	1.72	0.15	0.36	1.14
97XX1278	20	5544-LP30108-15	3.66	0.12	1.21	27.44	0	30.2	32.37	1.49	0.53	0.49	1.15
97XX1278	21	5544-LP30108-15	3.58	0.11	1.51	29.81	0	30.72	30.65	1.16	0.4	0.5	0.96
97XX1278	23	5544-LP30108-15	3.69	0.11	1.42	30.05	0	32.28	27.41	1.65	0.38	0.54	1.19
97XX1278	24	5544-LP30108-15	3.56	0.11	1.31	30.25	0	28.64	31.46	1.43	0.48	0.48	1.11

Table 11

CYCLE ID	SPL NO	STRAIN ID	16:0	16:1	18:0	18:1	18:2_Δ6,9	18:2_Δ9,12	18:3_Δ6,9, 12	18:3_Δ9,12, 15	18:4	20:0	20:1
97XX1278	25	5544-LP30108-15	4.41	0.22	2.08	15.05	0	23.77	49.51	1.18	0.96	0.87	0.85
97XX1278	26	5544-LP30108-15	3.75	0.14	1.59	23.55	0	27.91	38.8	1.39	0.61	0.59	0.97
97XX1278	27	5544-LP30108-15	3.67	0.11	1.9	26.07	0	31.1	33.16	1.08	0.49	0.65	0.97
97XX1278	28	5544-LP30108-15	3.82	0.11	1.54	21.27	0	29.07	39.69	1.47	0.7	0.58	0.86
97XX1278	29	5544-LP30108-15	3.65	0.14	1.27	45.84	0	43.38	1	2.33	0.02	0.42	1.27
97XX1278	30	5544-LP30108-15	3.59	0.12	1.19	30.41	0	30.68	30.37	1.24	0.4	0.37	0.99
97XX1278	31	5544-LP30108-15	3.74	0.12	1.26	38.98	0	50.53	0.98	2.12	0.02	0.39	1.14
97XX1278	32	5544-LP30108-15	3.86	0.11	1.46	26.38	0	28.9	35.41	1.01	0.5	0.54	0.97

Example 11

Simultaneous expression of *M. alpina* $\Delta 5$ and $\Delta 6$ desaturases in *Brassica napus*

5 In order to produce arachadonic acid (ARA) in transgenic canola oil both $\Delta 5$ and $\Delta 6$ desaturase activities need to be introduced. In order to facilitate downstream characterization and breeding, it may be advantageous to have both activities encoded by a single T-DNA. The following example illustrates the simultaneous expression of $\Delta 5$ and $\Delta 6$ desaturases.

10 The Asp718 fragment of pCGN5528 containing the napin 5' regulatory region, the Ma29 coding region, and the napin 3' regulatory region was inserted into the Asp718 site of pCGN5138 to create pCGN5545. The NotI fragment of pCGN5536 containing the napin 5' regulatory region, the Ma524 coding region, and the napin 3' regulatory region was inserted into the NotI site of pCGN5545
15 to create pCGN5546. The expression modules were oriented in such a way that the direction of transcription from Ma524 and Ma29 and the nptII marker is the same.

 PCGN5546 was introduced into *Brassica napus* cv.LP30108 via
20 *Agrobacterium* mediated transformation. Mature selfed T2 seeds were collected from 30 independent LP30108 transformation events that were grown in the greenhouse. The fatty acid composition of 20-seed pools was analyzed by GC. The results are shown in Table 12. All the lines show expression of both desaturases as evidenced by the presence of $\Delta^{5,9}$ 18:2 (as seen in pCGN5531 plants) and $\Delta^{6,9}$ 18:2 and GLA (as seen in pCGN5538 plants)

25 .

Table 12

fatty acid analysis of 20-seed pools of mature T2 seeds from 5546-LP30108 events

STRAIN ID	16:0	16:1	18:0	18:1	18:2_Δ5,9	18:2_Δ6,9	18:2_Δ9,12	18:3_Δ6,9, 12	18:3_Δ9,12, 15	18:4	20:0	20:1
5546-LP30108-1	4.88	0.33	2.28	57.2	4.68	6.08	7.36	12.29	1.38	0.85	0.84	1.22
5546-LP30108-2	4.01	0.14	2.22	66.04	2.73	1.33	12.6	6.45	1.41	0.32	0.75	1.2
5546-LP30108-3	4.29	0.15	2.55	68.89	0.44	0.58	16.97	1.66	1.6	0.11	0.88	1.22
5546-LP30108-4	4.24	0.14	2.6	70.48	0.73	0.52	14.28	2.61	1.42	0.14	0.96	1.26
5546-LP30108-5	3.52	0.15	2.01	60.3	1.72	0.95	16.92	9.88	1.66	0.39	0.68	1.26
5546-LP30108-6	4.05	0.17	2.24	61.29	1.98	0.4	18.87	6.28	2	0.34	0.7	1.24
5546-LP30108-7	4.74	0.21	2.49	64.5	2.25	1.18	10.03	9.73	1.35	0.52	0.97	1.28
5546-LP30108-8	4.24	0.14	2.82	63.92	1.9	1.5	11.67	9.29	1.44	0.43	0.89	1.19
5546-LP30108-9	3.8	0.13	2.15	65.75	2.3	0.16	14.92	6.32	1.57	0.24	0.75	1.35
5546-LP30108-10	4.28	0.17	1.55	58.8	1.1	0.12	22.95	5.97	2.24	0.22	0.6	1.35
5546-LP30108-11	4.25	0.15	1.82	63.68	1.01	0.22	19.42	4.96	1.81	0.2	0.67	1.23
5546-LP30108-12	3.95	0.14	2.36	66.9	1.12	0.01	19.42	1.59	1.77	0.04	0.8	1.21
5546-LP30108-13	4.18	0.16	2.17	66.91	1.36	0.02	18.84	1.99	1.74	0.05	0.77	1.15
5546-LP30108-14	4.74	0.26	1.82	65.29	1.25	0.27	16.77	5.3	1.59	0.25	0.71	1.32
5546-LP30108-15	4.3	0.23	2.54	65.65	1.67	0.59	13.15	7.22	1.54	0.36	0.88	1.3
5546-LP30108-16	4.05	0.17	2.75	64.13	2.56	2.8	9.56	9.31	1.34	0.53	0.92	1.28

Table 12

fatty acid analysis of 20-seed pools of mature T2 seeds from 5546-LP30108 events

STRAIN ID	16:0	16:1	18:0	18:1	18:2_Δ5,9	18:2_Δ6,9	18:2_Δ9,12	18:3_Δ6,9, 12	18:3_Δ9,12, 15	18:4	20:0	20:1
5546-LP30108-17	4.06	0.13	2.85	65.76	2.09	1.92	9.65	9.1	1.23	0.45	0.92	1.22
5546-LP30108-18	4.16	0.25	2.14	60.68	1.43	0.02	24.02	2.62	2.11	0.09	0.69	1.26
5546-LP30108-19	5.77	0.37	2.15	56.11	1.6	0.33	19.34	9.16	2.37	0.46	0.73	1.05
5546-LP30108-20	5.03	0.36	2.34	61.05	1.55	0.35	17.21	6.96	2.24	0.39	0.77	1.22
5546-LP30108-21	4.52	0.3	2.71	62.14	1.33	0.23	17.62	6.44	1.88	0.28	0.88	1.15
5546-LP30108-22	5.91	0.44	2.15	60.12	1.41	0.36	17.04	7.75	1.97	0.36	0.78	1.07
5546-LP30108-23	4.28	0.22	2.44	66.19	0.93	0.11	17.03	4.37	1.67	0.17	0.82	1.25
5546-LP30108-24	4.92	0.33	2.68	62.6	1.32	0.36	16.89	5.82	2.05	0.3	0.95	1.19
5546-LP30108-25	5.42	0.72	3.15	47.47	2.66	4.21	13.51	16.31	2.14	0.99	1.18	1.37
5546-LP30108-26	3.85	0.22	2.78	65.02	1.05	0.05	18.35	4.36	1.67	0.12	0.82	1.18
5546-LP30108-27	3.86	0.15	2.76	65.17	1.11	0.78	16.24	5.21	1.53	0.25	0.93	1.3
5546-LP30108-28	5.29	0.42	1.81	49.12	1.07	0.09	30.52	5.21	3.57	0.44	0.67	1.23
5546-LP30108-29	4.4	0.2	2.38	65.95	1.05	0.28	16.31	4.85	1.64	0.19	0.85	1.26
5546-LP30108-30	3.99	0.19	2.55	67.47	0.83	0.11	17.02	3.18	1.68	0.13	0.83	1.23

Example 12

Simultaneous expression of *M. alpina* $\Delta 5$, $\Delta 6$ and $\Delta 12$ desaturases in *Brassica napus*

5 In order to achieve optimal production of ARA in transgenic canola oil both the $\Delta 6$ and $\Delta 12$ desaturase activities may need to be present in addition to the $\Delta 5$ activity. In order to facilitate downstream characterization and breeding, it may be advantageous to have all of these activities encoded by a single T-DNA. The following example illustrates the simultaneous expression of $\Delta 5$, $\Delta 6$ and $\Delta 12$ desaturases.

10 The HindIII fragment of pCGN5528 containing the napin 5' regulatory region, the Ma29 coding region, and the napin 3' regulatory region was inserted into the HindIII site of pCGN5544 to create pCGN5547. The expression modules were oriented in such a way that the direction of transcription from

15 Ma29, Ma524, Ma648 and the nptII marker is the same.

 PCGN5547 was introduced into *Brassica napus* cv.LP30108 via *Agrobacterium* mediated transformation. Mature selfed T2 seeds were collected from 30 independent LP30108 transformation events that were grown in the greenhouse. The fatty acid composition of 20-seed pools was analyzed by GC.

20 The results are shown in Table 13. Twenty-seven of the lines show significant accumulation of GLA and in general the levels of GLA observed are higher than those seen in the 5546 plants that did not contain the $\Delta 12$ desaturase. The $\Delta 12$ desaturase appears to be active in most lines as evidenced by the lack of detectable $\Delta 6,9$ 18:2 and elevated 18:2 levels in most plants. Small amounts of

25 $\Delta 5,9$ 18:2 are seen in the 5547 plants, although the levels are generally less than those observed in the 5546 plants. This may be due to the presence of the $\Delta 12$ desaturase which efficiently converts the 18:1 to 18:2 before it can be desaturated at the $\Delta 5$ position.

Table 13

fatty acid analysis of 20-seed pools of mature T2 seeds from 5547-LP30108 events

STRAIN ID	12:0	16:0	16:1	18:0	18:1	18:2_Δ5, 9	18:2_Δ6,9	18:2_Δ9,12	18:3_Δ6,9, 12	18:3_Δ9,12, 15	20:0	20:1	22:1	22:2	
5547-LP30108-1	0.0	5.38	0.3	2.23	64.12	0.01	0	22.67	0.44	2.17	0.07	0.82	1.11	0.03	0
5547-LP30108-2	0.1	4.45	0.13	2.29	51.57	0.16	0	33.85	3.18	1.74	0.03	0.78	1.02	0.03	0.02
5547-LP30108-3	0.0	4.18	0.12	2.03	59.61	0.03	0	29.44	0.44	1.64	0	0.75	1.15	0.03	0.01
5547-LP30108-4	0.0	4.35	0.15	2.29	50.59	0.12	0.01	37.31	0.85	1.86	0.02	0.78	1.02	0.02	0.01
5547-LP30108-5	0.0	4.59	0.14	1.83	49	0.25	0.01	31.65	8.16	1.86	0.13	0.68	1.04	0.02	0
5547-LP30108-6	0.0	4.11	0.15	2.53	44.3	0.13	0	28.12	15.89	1.94	0.28	0.82	1.13	0	0
5547-LP30108-7	0.0	4.27	0.15	2.55	39.18	0.12	0.02	27	21.72	1.87	0.45	0.89	1.08	0	0
5547-LP30108-8	0.0	4.3	0.14	2.92	42.83	0.26	0	30.81	14.51	1.49	0.22	0.89	1.06	0	0
5547-LP30108-9	0.0	4.46	0.17	3.13	44.51	0	0	30.12	12.87	1.76	0.22	0.98	1.12	0.01	0
5547-LP30108-10	0.0	4.28	0.11	2.62	41.44	0.28	0	30.89	16.28	1.45	0.21	0.82	1.06	0	0
5547-LP30108-11	0.0	4.47	0.17	2.43	26.96	0.48	0	34.44	25.01	2.14	0.63	0.84	0.99	0	0
5547-LP30108-12	0.0	4.36	0.16	2.68	42.2	0.17	0	29.78	15.93	1.83	0.27	0.88	1.06	0	0
5547-LP30108-13	0.0	4.87	0.19	2.81	21.7	0.53	0	32.83	30.54	2.04	0.8	1	0.89	0.02	0.01
5547-LP30108-14	0.0	4.61	0.25	2.6	54	0	0	32.98	0.5	2.46	0.03	0.86	1.14	0	0
5547-LP30108-15	0.0	4.07	0.14	2.98	37.09	0.14	0.01	29.01	21.55	1.66	0.38	1.06	1.11	0	0

Table 13

fatty acid analysis of 20-seed pools of mature T2 seeds from 5547-LP30108 events

STRAIN ID	12:0	16:0	16:1	18:0	18:1	18:2_Δ5, 9	18:2_Δ6,9	18:2_Δ9,12	18:3_Δ6,9, 12	18:3_Δ9,12, 15	18:4	20:0	20:1	22:1	22:2	
5547-LP30108-16	0.0	3.63	0.13	2.12	64.69	0	0	0	24.21	0.15	2.04	0	0.82	1.56	0.02	0
5547-LP30108-17	0.0	3.85	0.18	2.22	67.22	0.01	0	0	21.25	0	2.27	0	0.83	1.53	0	0
5547-LP30108-18	0.0	5.46	0.19	2.87	41.83	0.1	0.04	0	22.76	21.45	1.72	0.48	1.06	1.23	0	0
5547-LP30108-19	0.0	4.33	0.12	2.73	50.31	0.07	0	0	24.77	12.72	1.62	0.21	1.04	1.29	0	0.01
5547-LP30108-20	0.0	4.22	0.12	2.91	46.33	0.25	0	0	26.87	14.65	1.61	0.22	0.98	1.18	0	0
5547-LP30108-21	0.0	4.38	0.17	2.37	55.37	0	0	0	32.59	0.53	1.85	0.03	0.83	1.23	0	0
5547-LP30108-22	0.0	5.5	0.18	2.71	41.93	0.1	0.19	0	24.19	20.14	1.76	0.45	0.94	1.21	0	0
5547-LP30108-23	0.0	4.03	0.16	2.17	68.44	0	0	0	20.09	0	2.19	0.02	0.83	1.46	0	0
5547-LP30108-24	0.0	4.19	0.17	2.72	49.31	0	0	0	30.38	8.64	1.85	0.13	0.86	1.16	0	0
5547-LP30108-25	0.0	4.04	0.17	2.1	70.48	0	0	0	18.04	0.05	2.09	0	0.86	1.54	0	0
5547-LP30108-26	0.0	4.74	0.22	3.2	26.74	0.33	0	0	30.05	28.95	2.02	0.78	1.08	0.99	0	0
5547-LP30108-27	0.0	4.29	0.18	2.23	52.49	0	0	0	28.48	7.36	1.91	0.13	0.87	1.37	0	0
5547-LP30108-28	0.0	4.36	0.17	3	44.35	0.2	0	0	29.59	13.39	1.91	0.23	0.96	1.17	0	0
5547-LP30108-29	0.0	4.32	0.17	2.94	52.53	0.05	0	0	33.88	0.91	2.34	0.01	0.97	1.23	0	0
5547-LP30108-30	0.0	4.07	0.14	2.89	45.13	0.01	0	0	29.06	13.96	1.71	0.2	0.94	1.2	0.01	0

Example 13

Stereospecific Distribution of $\Delta 6$ -Desaturated Oils

This experiment was designed to investigate the stereospecific distribution of the $\Delta 6$ -desaturated oils in seeds expressing pCGN5538 (Ma 524 cDNA). Three seed samples were used:

- 1) Non-transformed *B. napus* cv. LP004 seeds (control)
- 2) Segregating T2 seeds of pCGN5538-LP004-19
- 3) Segregating T2 seeds of pCGN5538-LP004-29

The following protocol was used for the analysis:

1. Seed Oil Extraction

Fifty seeds were placed in a 12 x 32 mm vial and crushed with a glass rod. 1.25 mL hexane was added and the mixture was vortexed. The seeds were extracted overnight on a shaker. The extract was then filtered through a 0.2 micron filter attached to a 1cc syringe. The extract was then dried down under nitrogen. The resulting oil was used for digestion and derivatization of the whole oil sample.

2. Digestion

A. Liquid Oil Digestion

The stock lipase (from *Rhizopus arrhizus*, Sigma, L4384) was diluted to approximately 600,000 units/mL with a goal of obtaining 50% digestion of the TAG. The stock lipase is maintained at 4 degrees C and placed on ice. The amount of reagents may be adjusted according to the amount of oil to be digested.

The following amounts are based on a 2.0 mg extracted oil sample. In a 12 x 32 mm screw cap vial the following were added: 2.0 mg oil, 200 μ L 0.1 M tris HCl pH 7, 40 μ L 2.2 w/v% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 100 μ L 0.05 w/v % bile salts. The material was vortexed and sonicated to disperse the oil. Twenty μ L of diluted lipase was added and the mixture was vortexed continuously for 1.0

minute at room temperature. A white precipitate formed. The reaction was stopped with 100 uL 6M HCl and vortexing. Five hundred uL $\text{CHCl}_3:\text{CH}_3\text{OH}$ (2:1) was added and the mixture was vortexed and held on ice while reaining digestions were carried out. Samples were vortexed again and centrifuged briefly to sharpen layers. The lower layer containing digest products was removed with a pasteur pipette and placed in a 12 x 32 mm crimp cap vial. The material was then re-extracted with 300 μL CHCl_3 , vortexed, centrifuged, and combined with the lower layers. The digest products were kept on ice as much as possible. HPLC separation is performed as soon as possible after digestion to minimize acyl migration.

B. Solid Fat Digestion

The procedure for liquid oil digestion described above was followed except that 20 μL 11:0 methyl ester is added to 2.0 mg solid fat.

3. HPLC Separation

The digestion products were dried down in chloroform to approximately 200 μL . Each sample was then transferred into an insert in an 8 x 40 mm shell vial and 30 μL was injected for HPLC analysis.

The high performance liquid chromatographic system was equipped with a Varex ELSD IIA evaporative light scattering detector with tube temperature at 105°C and nitrogen gas flow at 40 mL/min; a Waters 712 Wisp autosampler, three Beckman 114M Solvent Delivery Modules; a Beckman 421A controller, a Rheodyne pneumatically actuated stream splitter; and a Gilson micro fractionator. The chromatography column is a 220 x 4.6 mm, 5 micron normal phase silica cartridge by Brownlee.

The three solvents used were:

A= hexane:toluene 1:1

B= toluene: ethyl acetate 3:1

C= 5% formic acid in ethyl acetate

The gradient profile was as follows:

Time (min)	Function	Value	Duration
0 flow	2.0 mL/min		
0 % B	10		
0 % C	2		
2 % C	25		6 min
14.0 % C	2		1 min
15.0	End program		

A chromatographic standard mixture is prepared in hexane:toluene 1:1 containing the following:

- 0.2 mg/mL triglyceride 16:0
- 5 2.0 mg/mL 16:0 Free Fatty Acid
- 0.2 mg/mL di16:0 mixed isomers (1,2-diacylglycerol and 1,3-diacylglycerol)
- 0.2 mg/mL 3-mono acylglycerol 16:0
- 0.2 mg/mL 2-mono acylglycerol 16:0

10 For each sample, the fraction containing the 2-mag peak is collected automatically by method controlled timed events relays. A time delay is used to synchronize the detector with the collector's emitter. The 2-mag peaks are collected and the fractions are evaporated at room temperature overnight.

15 The *sn*-2 composition results rely on minimization of acyl migration. Appearance of 1-monoacylglycerol and/or 3-monoacylglycerol peaks in the chromatograph means that acyl migration has occurred.

4. Derivatization

20 To derivatize the whole oil, 1.0 mg of the extracted whole oil was weighed into a 12 x 32 mm crimp cap vial. One mL toluene was then added. The sample is then vortexed and a 50 μ L aliquot was removed for derivatization. To the dried down 2-mag samples, 50 μ L toluene was added. To both the whole oil and 2-mag fractions 105 μ L H₂SO₄/CH₃OH @ 8.76 wt% is added. The cap was tightly capped and the sample is refluxed for 1 hour at 95 degrees C. The sample was allowed to cool and 500 μ L 10 w/v % NaCl in

water and 60 uL heptane was added. The organic layer was removed and inserted in a 12 x 32 mm crimp cap vial.

5. GLC Analysis

5 A Hewlett Packard model 6890 GC equipped with a split/splitless capillary inlet, FID detector, 6890 series autosampler and 3392A Alpha Omega integrator is set up for the capillary column as follows:

A. Supelco Omegawax 250, 30 m length, 0.25 mm id, 0.25 um film thickness

10	injection port:	260 C
	detector:	270 C
	initial temp:	170 C
	initial time:	1.5 min
	rate:	30 deg/min
15	final temp:	245 C
	final time:	6.5 min
	injection vol:	1.5 uL
	head pressure:	25 psi
	split ratio:	30
20	carrier gas:	He
	make-up gas:	N ₂
	FID gas:	H + air

Percent compositions of fatty acid methyl esters are calculated as mole percents. For carbon chain lengths less than 12, the use of theoretical or
25 empirical response factors in the area percent calculation is desirable.

6. Calculations

The mean distribution of each acyl group at each *sn*-1 and *sn*-3 position was calculated.

mean *sn*-1 and *sn*-3 composition = (3 WO comp - MAG comp) / 2

5 WO = whole oil

MAG= monoacylglycerol

The results of this analysis are presented in Table 14. The GLA and $\Delta^{6,9}$ 18:2 are evenly distributed between the *sn*-2 and *sn*-1, 3 positions. This analysis can not discriminate between fatty acids in the *sn*-1 vs. *sn*-3 positions.

Table 14

	16:0	16:1	18:0	18:1	18:2_Δ6,9	18:2	18:3_Δ6,9,12	8:3	18:4	20:0	20:1
Control											
sn2 composition	1.23	0.15	0.37	64.77	0.00	29.45	0.06	2.01	0.00	0.21	0.57
whole oil composition	4.33	0.20	3.32	69.29	0.18	18.51	0.00	1.35	0.06	0.91	1.17
mean sn1, sn3 composition*	5.88	0.23	4.80	71.55	0.27	13.04	-0.03	1.02	0.09	1.26	1.47
5538-19											
sn2 composition	1.65	0.27	4.12	57.21	5.61	14.55	12.45	1.38	0.32	0.43	1.00
whole oil composition	5.44	0.33	4.09	57.51	4.53	10.57	13.16	1.03	0.50	1.07	1.07
mean sn1, sn3 composition*	7.34	0.36	4.08	57.66	3.99	8.58	13.52	0.86	0.59	1.39	1.11
5538-29											
sn2 composition	1.24	0.27	1.56	56.35	6.35	17.85	12.99	1.60	0.38	0.14	0.40
whole oil composition	4.96	0.32	3.73	54.92	4.99	12.11	13.66	1.10	0.50	0.99	1.11
mean sn1, sn3 composition*	6.82	0.35	4.82	54.21	4.31	9.24	14.00	0.85	0.56	1.42	1.47
*calculated from the mag and whole oil composition for each analyte											

Example 14

Fatty Acid Compositions of Transgenic Plants

$\Delta 5$ and $\Delta 6$ transgenic plants were analyzed for their fatty acid content.

The following protocol was used for oil extraction:

- 5 1. About 400 mg of seed were weighed out in duplicate for each sample.
2. The seeds were crushed in a mortar and pestle. The mortar and pestle was rinsed twice with 3ml (2:1) (v:v) CHCl_3 : CH_3OH /MeOH. An additional 6 ml (2:1) was added to
10 the 20ml glass vial (oil extracted in 12ml total 2:1).
3. Samples were vortexed and placed on an orbital shaker for 2 hours with occasional vortexing.
4. 5ml of 1M NaCl was added to each sample. Sample was vortexed then spun in centrifuge at 2000rpm for 5 minutes.
15 Lower phase was drawn off using a pasteur pipette.
5. Upper phase was re-extracted with an additional 5ml. Sample was vortexed then spun in centrifuge at 2000 rpm for 5 minutes. The lower phase was drawn off using a pasteur pipette and added to previous lower phase.
- 20 6. CHCl_3 : CH_3OH /MeOH was evaporated under nitrogen using evaporative cooling. Vial containing extracted oil was sealed under nitrogen. Between 120mg- 160mg oil was extracted for each sample.

25 For GC-MS analysis, fatty acid methyl esters were dissolved in an appropriate volume of hexane and analyzed using a Hewlett-Packard 5890 Series II Plus gas chromatograph (Hewlett Packard, Palo Alto, CA) equipped with a 30 m x 0.32 mm i.d. Omegawax 320 fused silica capillary column (Supelco, Bellefonte, PA) and a Hewlett-Packard 5972 Series mass selective detector. Mass spectra were interpreted by comparison to the mass spectra in

NIST/EPA/NIH Chemical Structure Database using a MS Chem Station
(#G1036A) (Hewlett Packard).

Transgenic line 5531-6 was analyzed in duplicate (A, B) and compared
to control line LP004-6. The fatty acid profile results are shown in Table 15.

- 5 Transgenic line 5538-19 was analyzed in duplicate (A, B) and compared
to control line LP004-6. The fatty acid profile results are shown in Table 16.

Table 15
Fatty Acid Profile

	CONTROL	CONTROL	TRANSGENIC	TRANSGENIC
	LP004-6A	LP004-6B	5531-6A	5531-6B
	LRL-2043	LRL-2044	LRL-2042	LRL-2045
	001f0102.d	001f0103.d	001f0101.d	001f0104.d
C12:0				
C13:0				
C14:0		0.053		0.061
C14:1				
C15:0 isomer				
C15:0				
C16:0	4.107	4.034	4.257	4.224
C16:1	0.181	0.173	0.200	0.199
C16:2	0.061	0.065	0.081	0.060
C17:0				
C16:3	0.244	0.246	0.155	0.151
C16:4				
C18:0	2.608	2.714	3.368	3.417
C18:1w9	65.489	66.454	59.529	59.073
C18:1w7	2.297	2.185	2.388	2.393
C18:2 5,9			6.144	6.269
C18:2w6	19.828	18.667	18.872	19.059
C18:3 5,9,12			0.469	0.496
C18:3w6		0.060		
C18:3w3	1.587	1.578	1.428	1.418
C18:4w6				
C18:4w3				
C20:0	0.962	0.998	1.009	1.022
C20:1w11	1.336	1.335	1.058	1.065
C20:1w9				
C20:1w7			0.076	0.080
C20:2w6	0.073	0.073		0.052
C20:3w6				

Table 15
Fatty Acid Profile

	CONTROL	CONTROL	TRANSGENIC	TRANSGENIC
	LP004-6A	LP004-6B	5531-6A	5531-6B
	LRL-2043	LRL-2044	LRL-2042	LRL-2045
	001f0102.d	001f0103.d	001f0101.d	001f0104.d
C20:4w6				
C20:3w3				
C20:4w3				
C20:5w3				
C22:0(1:000)	0.542	0.558	0.463	0.467
C22:1w11		0.038		
C22:1w9				
C22:1w7		0.034		
C21:5				
C23:0		0.029		
C22:4w6				
C22:5w6				
C22:5w3				
C24:0	0.373	0.391	0.280	0.283
C22:6w3	0.314	0.317	0.223	0.212
C24:1w9				
TOTAL	100.00	100.00	100.00	100.00

Table 16
Fatty Acid Profile

	5538-19A	5538-19B	LP004-6A	LP004-6B
	TRANSGENIC	TRANSGENIC	CONTROL	CONTROL
	LRL-2166	LRL-2167	LRL-2168	LRL-2169
C6:0	0.004	0.005		
C8:0	0.007	0.007	0.004	0.005
C10:0	0.012	0.012	0.008	0.008
C12:0	0.020	0.020	0.011	0.012
C13:0				
C14:0	0.099	0.108	0.050	0.050
C14:1w5				
C15:0	0.059	0.068	0.017	0.019
C16:0	5.272	5.294	4.049	4.057
C16:1	0.350	0.417	0.197	0.208
C16:2	0.199	0.187	0.076	0.077
C17:0	0.092	0.089	0.078	0.077
C16:3	0.149	0.149	0.192	0.198
C16:4		0.010		
C18:0	3.815	3.771	2.585	2.638
C18:1	57.562	57.051	68.506	68.352
C18:2 (6,9)	4.246	4.022		
C18:2w6	10.900	11.589	19.098	19.122
C18:2w3	0.020	0.008	0.008	0.009
C18:3w6	12.565	12.595	0.013	0.015
C18:3w3	1.084	1.137	1.501	1.542
C18:4	0.017	0.013	0.011	0.008
C18:4	0.028	0.024		
C20:0	1.138	1.104	0.937	0.943
C20:1	1.115	1.085	1.330	1.327
C20:2w6	0.150	0.143	0.068	0.071
C20:3w6	0.026	0.025	0.014	0.012
C20:4w6				
C20:3w3				

Table 16
Fatty Acid Profile

	5538-19A	5538-19B	LP004-6A	LP004-6B
	TRANSGENIC	TRANSGENIC	CONTROL	CONTROL
	LRL-2166	LRL-2167	LRL-2168	LRL-2169
C20:4w3				
C20:5w3				
C22:0	0.506	0.484	0.535	0.539
C22:1	0.017	0.020	0.032	0.032
C21:5		0.040	0.030	0.031
C22:4w6	0.038	0.064	0.015	0.014
C22:5w6				
C22:5w3	0.023	0.018	0.021	0.017
C24:0	0.352	0.321	0.353	0.362
C22:6w3	0.009			
C24:1w9	0.129	0.121	0.260	0.255
TOTAL	100.00	100.00	100.00	100.00

Example 15

Combined Expression of $\Delta 6$ and $\Delta 12$ Desaturases in *B. napus* Achieved by Crossing

Plants containing either the $\Delta 6$ or the $\Delta 12$ desaturase were crossed and individual F1 half-seeds were analyzed for fatty acid composition by GC. Data from one such cross are given in Table 17. The parents for the cross were 5538-LP004-25-2-25 ($\Delta 6$ expressor) and 5542-SP30021-10-16 ($\Delta 12$ expressor). Reciprocal crosses were made and the results of 25 individual F1 seeds of each are shown in the table. Crosses are described such that the first parent indicated is the female. Both sets of crosses gave approximately the same results. Compared to the parents, the $\Delta^{6,9}$ 18:2 decreased, and the GLA increased. $\Delta^{9,12}$ 18:2 levels are increased in most of the F1's as well. Note that these are F1 seeds and only contain one set of each desaturase. In future generations and selection of events homozygous for each desaturase, the F2 GLA levels obtained may be even higher.

Combining traits by crossing may be preferable to combining traits on one T-DNA in some situations. Particularly if both genes are driven off of the same promoter (in this case napin), issues of promoter silencing may favor this approach over putting multiple cDNAs on one construct.

Alternatively, in some cases, combining multiple cDNAs on one T-DNA may be the method of choice. The results are shown in Table 17.

Table 17

STRAIN ID	16:0	16:1	18:0	18:1	18:2_Δ6,9	18:2_Δ9,12	18:3_Δ6,9, 12	18:3_Δ9,12, 11	20:0	20:1
5538-LP004-25-2-25	4.23	0.13	2.4	61.78	8.77	6.34	11.58	0.92	0	0
5542-SP30021-10-16	4.09	0.1	2.03	38.4	0	41.88	0	11.06	0.02	0.75
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.9	0.04	2.31	38.58	0	27.91	20.94	2.67	0.65	0.92
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.5	0.04	1.88	36.24	0	28.68	22.54	3.36	0.85	0.78
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.51	0.03	1.98	38.36	0	29.48	19.95	3.06	0.68	0.79
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.95	0.04	1.86	38.65	0	28.08	20.81	2.92	0.75	0.76
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	4.26	0.05	2.44	40.25	0.01	28.81	18.08	2.74	0.53	0.88
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	4.13	0.04	2.33	34.48	0	26.73	26.2	2.32	0.75	0.9
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.8	0.04	2.15	38.34	0	28.95	20.64	2.63	0.65	0.81
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.96	0.05	1.59	36.43	0	29.05	21.85	3.47	0.86	0.68
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	4.04	0.04	2.5	37.75	0	27.23	22.89	1.95	0.55	0.99
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.53	0.04	1.8	34.88	0	29.17	23.42	3.42	0.9	0.74
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.43	0.04	1.89	37.12	0	29.52	20.91	3.35	0.8	0.79
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.58	0.03	2.55	39.54	0	28.81	19.34	2.44	0.54	0.98
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.53	0.03	2.33	39.26	0	29.07	19.5	2.61	0.59	0.91
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.4	0.02	2.41	45.53	0	28.94	13.71	2.51	0.37	0.91

Table 17

STRAIN ID	16:0	16:1	18:0	18:1	18:2_Δ6,9	18:2_Δ9,12	18:3_Δ6,9, 12	18:3_Δ9,12, 11	20:0	20:1
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.49	0.03	2.57	40.95	0	28.52	17.97	2.63	0.58	0.99
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.65	0.04	2.11	38.02	0	29.13	20.53	2.85	0.66	0.86
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.97	0.03	1.99	34.95	0.01	27.15	25.71	2.38	0.79	0.81
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.81	0.05	1.46	38.3	0	31.51	17.67	3.83	0.75	0.61
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.98	0.05	2.03	37.14	0	30.09	20.28	2.79	0.72	0.8
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	4.03	0.04	2.52	42.9	0	27.79	16.66	2.64	0.54	0.9
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	4.03	0.04	2.27	40.72	0	29.37	17.56	2.53	0.53	0.86
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.98	0.04	2.61	39.91	0	28.06	19.15	2.69	0.6	0.96
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.73	0.03	1.89	40.22	0	29.44	18.21	3	0.67	0.73
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	4.02	0.04	2.14	42.58	0	30.36	15.18	2.43	0.42	0.82
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4.14	0.06	2.23	30.67	0	30.38	25.47	3.12	0.91	0.9
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4.05	0.07	1.7	37.03	0.04	32.1	15.97	5.38	0.96	0.69
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4.01	0.07	1.58	38.02	0.05	33.65	13.92	5.15	0.89	0.66
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4.07	0.06	2.01	31.63	0.05	31.13	23.09	3.94	1.1	0.83
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4.03	0.05	1.94	31.88	0	30.98	23.71	3.45	0.99	0.82
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	3.92	0.06	1.71	35.77	0.03	33.15	16.39	5.28	0.98	0.68
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4.09	0.08	1.57	34.6	0.03	33.73	16.73	5.48	0.99	0.66

Table 17

STRAIN ID	16:0	16:1	18:0	18:1	18:2_Δ6,9	18:2_Δ9,12	18:3_Δ6,9, 12	18:3_Δ9,12, 11	18:4	20:0	20:1
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	3.94	0.07	1.59	34.03	0.04	31.35	19.76	5.29	1.22	0.67	1.28
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4.13	0.06	1.85	31.44	0.06	31.28	23.77	3.52	1.04	0.79	1.22
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4.14	0.06	1.96	31.11	0.04	31.88	23.3	3.6	1.01	0.82	1.27
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	3.98	0.07	1.58	35.06	0	32.06	18.1	5.33	1.12	0.67	1.28
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	3.89	0.06	1.59	32.51	0.05	29.44	22.91	5.33	1.54	0.67	1.25
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4	0.07	1.69	32.1	0.05	30.49	22.77	4.66	1.32	0.75	1.26
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4.06	0.05	1.93	30.77	0.07	28.37	27.21	3.37	1.19	0.84	1.25
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4.1	0.06	1.9	31.77	0.05	32.33	22.03	3.92	0.98	0.78	1.27
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	3.94	0.07	1.67	34.74	0.03	33.63	17.1	5.16	0.99	0.68	1.26
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	3.71	0.06	1.65	33.05	0	33.22	19.73	4.7	1.07	0.68	1.39
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	3.84	0.06	1.71	34.16	0.04	34.52	16.74	5.18	0.97	0.68	1.34
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4	0.07	1.66	34.97	0.07	33.08	17.07	5.27	1.1	0.67	1.28
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4.16	0.06	1.99	35.44	0.05	31.89	18.95	3.68	0.89	0.81	1.29
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4.05	0.08	1.46	33.49	0	31.96	18.81	6.2	1.32	0.61	1.28
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4.2	0.06	1.93	35.06	0.06	33.69	17.38	4	0.86	0.78	1.21
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4.07	0.06	1.74	36	0.06	32.18	17.86	4.32	0.96	0.73	1.27
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4.11	0.05	2.24	29.64	0.04	28.64	27.94	3.06	1.12	0.97	1.26

Example 16

Expression of *M. alpina* desaturases in soybean

The *M. alpina* desaturases can be used to drive production of GLA and other PUFAs in soybean by use of the following expression constructs. Two means by which exogenous DNA can be inserted into the soybean genome are *Agrobacterium* infection or particle gun. Particle gun transformation is disclosed in U.S. patent 5,503,998. Plants can be selected using a glyphosate resistance marker (4, 971, 908). *Agrobacterium* transformation of soybean is well established to one of ordinary skill in the art.

For seed specific expression, the coding regions of the desaturase cDNAs are placed under control of the 5' regulatory region of *Glycine max* alpha-type beta conglycinin storage protein gene. The specific region that can be used is nucleotides 78-921 of gi 169928 (Doyle, J.J., Schuler, M.A., Godette, W.D., Zenger, V., Beachy, R.N., and Slightom, J.L., 1986 J. Biol. Chem. 261 (20), 9228-9238). The 3' regulatory region that can be used is from the pea ribulose 1,5 biphosphate carboxylase/oxygenase small subunit (*rbcS*) gene. The specific sequences to be used are nucleotides 1-645 of gi 169145 (Hunt, A.G. 1988 DNA 7: 329-336).

Since soybean seeds contain more 18:2, and perhaps more endogenous $\Delta 12$ desaturase activity than *Brassica*, the effect of the *Mortierella* $\Delta 12$ desaturase on achieving optimal GLA levels can be tested as follows. A construct containing the $\Delta 6$ cDNA can be used to see if $\Delta^{6,9}$ 18:2 is produced along with GLA. A construct containing the $\Delta 12$ desaturase can be used to see if the amount of 18:2 can be increased in soybean. A construct containing both the $\Delta 6$ and $\Delta 12$ desaturases can be used to produce optimal levels of GLA. Alternatively, plants containing each of the single desaturases may be crossed if necessary to combine the genes.

Similar constructs may be made to express the $\Delta 5$ desaturase alone, or in combination with $\Delta 12$ and/or $\Delta 6$ desaturases.

Example 17

Human Desaturase Gene Sequences

Human desaturase gene sequences potentially involved in long chain polyunsaturated fatty acid biosynthesis were isolated based on homology
5 between the human cDNA sequences and *Mortierella alpina* desaturase gene sequences. The three conserved "histidine boxes" known to be conserved among membrane-bound desaturases were found. As with some other membrane-bound desaturases the final HXXHH histidine box motif was found to be QXXHH. The amino acid sequence of the putative human desaturases
10 exhibited homology to *M. alpina* $\Delta 5$, $\Delta 6$, $\Delta 9$, and $\Delta 12$ desaturases.

The *M. alpina* $\Delta 5$ desaturase and $\Delta 6$ desaturase cDNA sequences were used to search the LifeSeq database of Incyte Pharmaceuticals, Inc., Palo Alto, California 94304. The $\Delta 5$ desaturase sequence was divided into fragments; 1) amino acid no. 1-150, 2) amino acid no. 151-300, and 3) amino acid no. 301-
15 446. The $\Delta 6$ desaturase sequence was divided into three fragments; 1) amino acid no. 1-150, 2) amino acid no. 151-300, and 3) amino acid no. 301-457. These polypeptide fragments were searched against the database using the "tblastn" algorithm. This algorithm compares a protein query sequence against a nucleotide sequence database dynamically translated in all six reading frames
20 (both strands).

The polypeptide fragments 2 and 3 of *M. alpina* $\Delta 5$ and $\Delta 6$ have homologies with the CloneID sequences as outlined in Table 18. The CloneID represents an individual sequence from the Incyte LifeSeq database. After the "tblastn" results have been reviewed, Clone Information was searched with the
25 default settings of Stringency of ≥ 50 , and Productscore ≤ 100 for different CloneID numbers. The Clone Information Results displayed the information including the ClusterID, CloneID, Library, HitID, Hit Description. When selected, the ClusterID number displayed the clone information of all the clones that belong in that ClusterID. The Assemble command assembles all of the
30 CloneID which comprise the ClusterID. The following default settings were

used for GCG (Genetics Computer Group, University of Wisconsin Biotechnology Center, Madison, Wisconsin 53705) Assembly:

	Word Size:	7
5	Minimum Overlap:	14
	Stringency:	0.8
	Minimum Identity:	14
	Maximum Gap:	10
	Gap Weight:	8
10	Length Weight:	2

GCG Assembly Results displayed the contigs generated on the basis of sequence information within the CloneID. A contig is an alignment of DNA sequences based on areas of homology among these sequences. A new sequence (consensus sequence) was generated based on the aligned DNA sequences within a contig. The contig containing the CloneID was identified, and the ambiguous sites of the consensus sequence was edited based on the alignment of the CloneIDs (see SEQ ID NO:31 - SEQ ID NO:35) to generate the best possible sequence. The procedure was repeated for all six CloneID listed in Table 18. This produced five unique contigs. The edited consensus sequences of the 5 contigs were imported into the Sequencher software program (Gene Codes Corporation, Ann Arbor, Michigan 48105). These consensus sequences were assembled. The contig 2511785 overlaps with contig 3506132, and this new contig was called 2535 (SEQ ID NO:37). The contigs from the Sequencher program were copied into the Sequence Analysis software package of GCG.

Each contig was translated in all six reading frames into protein sequences. The *M. alpina* $\Delta 5$ (MA29) and $\Delta 6$ (MA524) sequences were compared with each of the translated contigs using the FastA search (a Pearson

and Lipman search for similarity between a query sequence and a group of sequences of the same type (nucleic acid or protein)). Homology among these sequences suggest the open reading frames of each contig. The homology among the *M. alpina* $\Delta 5$ and $\Delta 6$ to contigs 2535 and 3854933 were utilized to
5 create the final contig called 253538a. Figure 9 is the FastA match of the final contig 253538a and MA29, and Figure 10 is the FastA match of the final contig 253538a and MA524. The DNA sequences for the various contigs are presented in SEQ ID NO:31 -SEQ ID NO:37 The various peptide sequences are shown in SEQ ID NO:38 - SEQ ID NO: 44.

10 Although the open reading frame was generated by merging the two contigs, the contig 2535 shows that there is a unique sequence in the beginning of this contig which does not match with the contig 3854933. Therefore, it is possible that these contigs were generated from independent desaturase like human genes.

15 The contig 253538a contains an open reading frame encoding 432 amino acids. It starts with Gln (CAG) and ends with the stop codon (TGA). The contig 253538a aligns with both *M. alpina* $\Delta 5$ and $\Delta 6$ sequences, suggesting that it could be either of the desaturases, as well as other known desaturases which share homology with each other. The individual contigs
20 listed in Table 18, as well as the intermediate contig 2535 and the final contig 253538a can be utilized to isolate the complete genes for human desaturases.

Uses of the Human Desaturases

These human sequences can be expressed in yeast and plants utilizing the procedures described in the preceding examples. For expression in
25 mammalian cells and transgenic animals, these genes may provide superior codon bias. In addition, these sequences can be used to isolate related desaturase genes from other organisms.

Table 18

Sections of the Desaturases	Clone ID from LifeSeq Database	Keyword
151-300 $\Delta 5$	3808675	fatty acid desaturase
301-446 $\Delta 5$	354535	$\Delta 6$
151-300 $\Delta 6$	3448789	$\Delta 6$
151-300 $\Delta 6$	1362863	$\Delta 6$
151-300 $\Delta 6$	2394760	$\Delta 6$
301-457 $\Delta 6$	3350263	$\Delta 6$

Example 185 **Identification of Homologues to *M. alpina* $\Delta 5$ and $\Delta 6$ desaturases**

A nucleic acid sequence that encodes a putative $\Delta 5$ desaturase was identified through a TBLASTN search of the expressed sequence tag databases through NCBI using amino acids 100-446 of Ma29 as a query. The truncated portion of the Ma29 sequence was used to avoid picking up homologies based on the cytochrome b5 portion at the N-terminus of the desaturase. The deduced amino acid sequence of an est from *Dictyostelium discoideum* (accession # C25549) shows very significant homology to Ma29 and lesser, but still significant homology to Ma524. The DNA sequence is presented as SEQ ID NO:45. The amino acid sequence is presented as SEQ ID NO:46.

15

Example 19**Identification of *M. alpina* $\Delta 5$ and $\Delta 6$ homologues in other PUFA-producing organisms**

To look for desaturases involved in PUFA production, a cDNA library was constructed from total RNA isolated from *Phaeodactylum tricornutum*. A plasmid-based cDNA library was constructed in pSPORT1 (GIBCO-BRL) following manufacturer's instructions using a commercially available kit (GIBCO-BRL). Random cDNA clones were sequenced and nucleic acid sequences that encode putative $\Delta 5$ or $\Delta 6$ desaturases were identified through BLAST search of the databases and comparison to Ma29 and Ma524 sequences.

One clone was identified from the *Phaeodactylum* library with homology to Ma29 and Ma524; it is called 144-011-B12. The DNA sequence is presented as SEQ ID NO:47. The amino acid sequence is presented as SEQ ID NO:48.

5

Example 20

Identification of *M. alpina* $\Delta 5$ and $\Delta 6$ homologues in other PUFA-producing organisms

To look for desaturases involved in PUFA production, a cDNA library was constructed from total RNA isolated from *Schizochytrium* species. A plasmid-based cDNA library was constructed in pSPORT1 (GIBCO-BRL) following manufacturer's instructions using a commercially available kit (GIBCO-BRL). Random cDNA clones were sequenced and nucleic acid sequences that encode putative $\Delta 5$ or $\Delta 6$ desaturases were identified through BLAST search of the databases and comparison to Ma29 and Ma524 sequences.

15

One clone was identified from the *Schizochytrium* library with homology to Ma29 and Ma524; it is called 81-23-C7. This clone contains a ~1 kb insert. Partial sequence was obtained from each end of the clone using the universal forward and reverse sequencing primers. The DNA sequence from the forward primer is presented as SEQ ID NO:49. The peptide sequence is presented as SEQ ID NO:50. The DNA sequence from the reverse primer is presented as SEQ ID NO:51. The amino acid sequence from the reverse primer is presented as SEQ ID NO:52.

20

Example 21

Nutritional Compositions

25

The PUFAs of the previous examples can be utilized in various nutritional supplements, infant formulations, nutritional substitutes and other nutrition solutions.

I. INFANT FORMULATIONS

A. Isomil® Soy Formula with Iron.

Usage: As a beverage for infants, children and adults with an allergy or sensitivity to cow's milk. A feeding for patients with disorders for which lactose should be avoided: lactase deficiency, lactose intolerance and galactosemia.

5 Features:

- Soy protein isolate to avoid symptoms of cow's-milk-protein allergy or sensitivity
- Lactose-free formulation to avoid lactose-associated diarrhea
- Low osmolality (240 mOsm/kg water) to reduce risk of osmotic diarrhea.

10

- Dual carbohydrates (corn syrup and sucrose) designed to enhance carbohydrate absorption and reduce the risk of exceeding the absorptive capacity of the damaged gut.

15

- 1.8 mg of Iron (as ferrous sulfate) per 100 Calories to help prevent iron deficiency.
- Recommended levels of vitamins and minerals.
- Vegetable oils to provide recommended levels of essential fatty acids.
- Milk-white color, milk-like consistency and pleasant aroma.

20

Ingredients: (Pareve, ®) 85% water, 4.9% corn syrup, 2.6% sugar (sucrose), 2.1% soy oil, 1.9% soy protein isolate, 1.4% coconut oil, 0.15% calcium citrate, 0.11 % calcium phosphate tribasic, potassium citrate, potassium phosphate monobasic, potassium chloride, mono- and diglycerides, soy lecithin, carrageenan, ascorbic acid, L-methionine, magnesium chloride, potassium phosphate dibasic, sodium chloride, choline chloride, taurine, ferrous sulfate, m-inositol, alpha-tocopheryl acetate, zinc sulfate, L-carnitine, niacinamide, calcium pantothenate, cupric sulfate, vitamin A palmitate, thiamine chloride hydrochloride, riboflavin, pyridoxine hydrochloride, folic

25

acid, manganese sulfate, potassium iodide, phylloquinone, biotin, sodium selenite, vitamin D₃ and cyanocobalamin.

B. Isomil® DF Soy Formula For Diarrhea.

Usage: As a short-term feeding for the dietary management of diarrhea
5 in infants and toddlers.

Features:

- First infant formula to contain added dietary fiber from soy fiber specifically for diarrhea management.
- Clinically shown to reduce the duration of loose, watery stools
10 during mild to severe diarrhea in infants.
- Nutritionally complete to meet the nutritional needs of the infant.
- Soy protein isolate with added L-methionine meets or exceeds an infant's requirement for all essential amino acids.
- Lactose-free formulation to avoid lactose-associated diarrhea.
- 15 • Low osmolality (240 mOsm/kg water) to reduce the risk of osmotic diarrhea.
- Dual carbohydrates (corn syrup and sucrose) designed to enhance carbohydrate absorption and reduce the risk of exceeding the absorptive capacity of the damaged gut.
- 20 • Meets or exceeds the vitamin and mineral levels recommended by the Committee on Nutrition of the American Academy of Pediatrics and required by the Infant Formula Act.
- 1.8 mg of iron (as ferrous sulfate) per 100 Calories to help prevent iron deficiency.
- 25 • Vegetable oils to provide recommended levels of essential fatty acids.

Ingredients: (Pareve, ©) 86% water, 4.8% corn syrup, 2.5% sugar (sucrose), 2.1% soy oil, 2.0% soy protein isolate, 1.4% coconut oil, 0.77% soy

fiber, 0.12% calcium citrate, 0.11 % calcium phosphate tribasic, 0.10% potassium citrate, potassium chloride, potassium phosphate monobasic, mono- and diglycerides, soy lecithin, carrageenan, magnesium chloride, ascorbic acid, L-methionine, potassium phosphate dibasic, sodium chloride, choline chloride, taurine, ferrous sulfate, m-inositol, alpha-tocopheryl acetate, zinc sulfate, L-carnitine, niacinamide, calcium pantothenate, cupric sulfate, vitamin A palmitate, thiamine chloride hydrochloride, riboflavin, pyridoxine hydrochloride, folic acid, manganese sulfate, potassium iodide, phylloquinone, biotin, sodium selenite, vitamin D₃ and cyanocobalamin.

10 **C. Isomil® SF Sucrose-Free Soy Formula With Iron.**

Usage: As a beverage for infants, children and adults with an allergy or sensitivity to cow's-milk protein or an intolerance to sucrose. A feeding for patients with disorders for which lactose and sucrose should be avoided.

Features:

- 15 • Soy protein isolate to avoid symptoms of cow's-milk-protein allergy or sensitivity.
- Lactose-free formulation to avoid lactose-associated diarrhea (carbohydrate source is Polycose® Glucose Polymers).
- Sucrose free for the patient who cannot tolerate sucrose.
- 20 • Low osmolality (180 mOsm/kg water) to reduce risk of osmotic diarrhea.
- 1.8 mg of iron (as ferrous sulfate) per 100 Calories to help prevent iron deficiency.
- Recommended levels of vitamins and minerals.
- 25 • Vegetable oils to provide recommended levels of essential fatty acids.
- Milk-white color, milk-like consistency and pleasant aroma.

Ingredients: (Pareve, ©) 75% water, 11.8% hydrolyzed cornstarch, 4.1% soy oil, 4.1% soy protein isolate, 2.8% coconut oil, 1.0% modified cornstarch,

0.38% calcium phosphate tribasic, 0.17% potassium citrate, 0.13% potassium chloride, mono- and diglycerides, soy lecithin, magnesium chloride, ascorbic acid, L-methionine, calcium carbonate, sodium chloride, choline chloride, carrageenan, taurine, ferrous sulfate, m-inositol, alpha-tocopheryl acetate, zinc sulfate, L-carnitine, niacinamide, calcium pantothenate, cupric sulfate, vitamin A palmitate, thiamine chloride hydrochloride, riboflavin, pyridoxine hydrochloride, folic acid, manganese sulfate, potassium iodide, phylloquinone, biotin, sodium selenite, vitamin D₃ and cyanocobalamin.

**D. Isomil® 20 Soy Formula With Iron Ready To Feed,
20 Cal/fl oz.**

Usage: When a soy feeding is desired.

Ingredients: (Pareve, ©) 85% water, 4.9% corn syrup, 2.6% sugar (sucrose), 2.1% soy oil, 1.9% soy protein isolate, 1.4% coconut oil, 0.15% calcium citrate, 0.11% calcium phosphate tribasic, potassium citrate, potassium phosphate monobasic, potassium chloride, mono- and diglycerides, soy lecithin, carrageenan, ascorbic acid, L-methionine, magnesium chloride, potassium phosphate dibasic, sodium chloride, choline chloride, taurine, ferrous sulfate, m-inositol, alpha-tocopheryl acetate, zinc sulfate, L-carnitine, niacinamide, calcium pantothenate, cupric sulfate, vitamin A palmitate, thiamine chloride hydrochloride, riboflavin, pyridoxine hydrochloride, folic acid, manganese sulfate, potassium iodide, phylloquinone, biotin, sodium selenite, vitamin D₃ and cyanocobalamin.

E. Similac® Infant Formula

Usage: When an infant formula is needed: if the decision is made to discontinue breastfeeding before age 1 year, if a supplement to breastfeeding is needed or as a routine feeding if breastfeeding is not adopted.

Features:

- Protein of appropriate quality and quantity for good growth; heat-denatured, which reduces the risk of milk-associated enteric blood loss.
- 5 • Fat from a blend of vegetable oils (doubly homogenized), providing essential linoleic acid that is easily absorbed.
- Carbohydrate as lactose in proportion similar to that of human milk.
- Low renal solute load to minimize stress on developing organs.
- 10 • Powder, Concentrated Liquid and Ready To Feed forms.

Ingredients: (©-D) Water, nonfat milk, lactose, soy oil, coconut oil, mono- and diglycerides, soy lecithin, ascorbic acid, carrageenan, choline chloride, taurine, m-inositol, alpha-tocopheryl acetate, zinc sulfate, niacinamid, ferrous sulfate, calcium pantothenate, cupric sulfate, vitamin A palmitate, 15 thiamine chloride hydrochloride, riboflavin, pyridoxine hydrochloride, folic acid, manganese sulfate, phylloquinone, biotin, sodium selenite, vitamin D₃ and cyanocobalamin.

F. Similac® NeoCare Premature Infant Formula With Iron

Usage: For premature infants' special nutritional needs after hospital 20 discharge. Similac NeoCare is a nutritionally complete formula developed to provide premature infants with extra calories, protein, vitamins and minerals needed to promote catch-up growth and support development.

Features:

- 25 • Reduces the need for caloric and vitamin supplementation. More calories (22 Cal/fl oz) than standard term formulas (20 Cal/fl oz).
- Highly absorbed fat blend, with medium-chain triglycerides (MCT oil) to help meet the special digestive needs of premature infants.
- Higher levels of protein, vitamins and minerals per 100 Calories to extend the nutritional support initiated in-hospital.

- More calcium and phosphorus for improved bone mineralization.

Ingredients: ©-D Corn syrup solids, nonfat milk, lactose, whey protein concentrate, soy oil, high-oleic safflower oil, fractionated coconut oil (medium-chain triglycerides), coconut oil, potassium citrate, calcium phosphate tribasic, calcium carbonate, ascorbic acid, magnesium chloride, potassium chloride, sodium chloride, taurine, ferrous sulfate, m-inositol, choline chloride, ascorbyl palmitate, L-carnitine, alpha-tocopheryl acetate, zinc sulfate, niacinamide, mixed tocopherols, sodium citrate, calcium pantothenate, cupric sulfate, thiamine chloride hydrochloride, vitamin A palmitate, beta carotene, riboflavin, pyridoxine hydrochloride, folic acid, manganese sulfate, phylloquinone, biotin, sodium selenite, vitamin D₃ and cyanocobalamin.

G. Similac Natural Care Low-Iron Human Milk Fortifier Ready To Use, 24 Cal/fl oz.

Usage: Designed to be mixed with human milk or to be fed alternatively with human milk to low-birth-weight infants.

Ingredients: ©-D Water, nonfat milk, hydrolyzed cornstarch, lactose, fractionated coconut oil (medium-chain triglycerides), whey protein concentrate, soy oil, coconut oil, calcium phosphate tribasic, potassium citrate, magnesium chloride, sodium citrate, ascorbic acid, calcium carbonate, mono- and diglycerides, soy lecithin, carrageenan, choline chloride, m-inositol, taurine, niacinamide, L-carnitine, alpha tocopheryl acetate, zinc sulfate, potassium chloride, calcium pantothenate, ferrous sulfate, cupric sulfate, riboflavin, vitamin A palmitate, thiamine chloride hydrochloride, pyridoxine hydrochloride, biotin, folic acid, manganese sulfate, phylloquinone, vitamin D₃, sodium selenite and cyanocobalamin.

Various PUFAs of this invention can be substituted and/or added to the infant formulae described above and to other infant formulae known to those in the art..

II. NUTRITIONAL FORMULATIONS

A. ENSURE®

Usage: ENSURE is a low-residue liquid food designed primarily as an oral nutritional supplement to be used with or between meals or, in appropriate amounts, as a meal replacement. ENSURE is lactose- and gluten-free, and is suitable for use in modified diets, including low-cholesterol diets. Although it is primarily an oral supplement, it can be fed by tube.

Patient Conditions:

- For patients on modified diets
- For elderly patients at nutrition risk
- For patients with involuntary weight loss
- For patients recovering from illness or surgery
- For patients who need a low-residue diet

Ingredients:

©-D Water, Sugar (Sucrose), Maltodextrin (Corn), Calcium and Sodium Caseinates, High-Oleic Safflower Oil, Soy Protein Isolate, Soy Oil, Canola Oil, Potassium Citrate, Calcium Phosphate Tribasic, Sodium Citrate, Magnesium Chloride, Magnesium Phosphate Dibasic, Artificial Flavor, Sodium Chloride, Soy Lecithin, Choline Chloride, Ascorbic Acid, Carrageenan, Zinc Sulfate, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Gellan Gum, Niacinamide, Calcium Pantothenate, Manganese Sulfate, Cupric Sulfate, Vitamin A Palmitate, Thiamine Chloride Hydrochloride, Pyridoxine Hydrochloride, Riboflavin, Folic Acid, Sodium Molybdate, Chromium Chloride, Biotin, Potassium Iodide, Sodium Selenate.

B. ENSURE® BARS

Usage: ENSURE BARS are complete, balanced nutrition for supplemental use between or with meals. They provide a delicious, nutrient-

rich alternative to other snacks. ENSURE BARS contain <1 g lactose/bar, and Chocolate Fudge Brownie flavor is gluten-free. (Honey Graham Crunch flavor contains gluten.)

Patient Conditions:

- 5 • For patients who need extra calories, protein, vitamins and minerals
- Especially useful for people who do not take in enough calories and nutrients
- For people who have the ability to chew and swallow
- Not to be used by anyone with a peanut allergy or any type of allergy to
10 nuts.

Ingredients:

Honey Graham Crunch -- High-Fructose Corn Syrup, Soy Protein-
Isolate, Brown Sugar, Honey, Maltodextrin (Corn), Crisp Rice (Milled Rice,
Sugar [Sucrose], Salt [Sodium Chloride] and Malt), Oat Bran, Partially
15 Hydrogenated Cottonseed and Soy Oils, Soy Polysaccharide, Glycerine, Whey
Protein Concentrate, Polydextrose, Fructose, Calcium Caseinate, Cocoa
Powder, Artificial Flavors, Canola Oil, High-Oleic Safflower Oil, Nonfat Dry
Milk, Whey Powder, Soy Lecithin and Corn Oil. Manufactured in a facility that
processes nuts.

20 **Vitamins and Minerals:**

Calcium Phosphate Tribasic, Potassium Phosphate Dibasic, Magnesium
Oxide, Salt (Sodium Chloride), Potassium Chloride, Ascorbic Acid, Ferric
Orthophosphate, Alpha-Tocopheryl Acetate, Niacinamide, Zinc Oxide, Calcium
Pantothenate, Copper Gluconate, Manganese Sulfate, Riboflavin, Beta-
25 Carotene, Pyridoxine Hydrochloride, Thiamine Mononitrate, Folic Acid, Biotin,
Chromium Chloride, Potassium Iodide, Sodium Selenate, Sodium Molybdate,
Phylloquinone, Vitamin D₃ and Cyanocobalamin.

Protein:

Honey Graham Crunch - The protein source is a blend of soy protein isolate and milk proteins.

5	Soy protein isolate	74%
	Milk proteins	26%

Fat:

Honey Graham Crunch - The fat source is a blend of partially hydrogenated cottonseed and soybean, canola, high oleic safflower, and corn oils, and soy lecithin.

10	Partially hydrogenated cottonseed and soybean oil	76%
	Canola oil	8%
	High-oleic safflower oil	8%
	Corn oil	4%
	Soy lecithin	4%

15 **Carbohydrate:**

Honey Graham Crunch - The carbohydrate source is a combination of high-fructose corn syrup, brown sugar, maltodextrin, honey, crisp rice, glycerine, soy polysaccharide, and oat bran.

20	High-fructose corn syrup	24%
	Brown sugar	21%
	Maltodextrin	12%
	Honey	11%
	Crisp rice	9%
	Glycerine	9%
25	Soy polysaccharide	7%
	Oat bran	7%

C. ENSURE® HIGH PROTEIN

Usage: ENSURE HIGH PROTEIN is a concentrated, high-protein liquid food designed for people who require additional calories, protein, vitamins, and minerals in their diets. It can be used as an oral nutritional supplement with or between meals or, in appropriate amounts, as a meal replacement. ENSURE HIGH PROTEIN is lactose- and gluten-free, and is suitable for use by people recovering from general surgery or hip fractures and by patients at risk for pressure ulcers.

Patient Conditions

- For patients who require additional calories, protein, vitamins, and minerals, such as patients recovering from general surgery or hip fractures, patients at risk for pressure ulcers, and patients on low-cholesterol diets

Features-

- Low in saturated fat
- Contains 6 g of total fat and < 5 mg of cholesterol per serving
- Rich, creamy taste
- Excellent source of protein, calcium, and other essential vitamins and minerals
- For low-cholesterol diets
- Lactose-free, easily digested

Ingredients:

Vanilla Supreme: -D Water, Sugar (Sucrose), Maltodextrin (Corn), Calcium and Sodium Caseinates, High-Oleic Safflower Oil, Soy Protein Isolate, Soy Oil, Canola Oil, Potassium Citrate, Calcium Phosphate Tribasic, Sodium Citrate, Magnesium Chloride, Magnesium Phosphate Dibasic, Artificial Flavor, Sodium Chloride, Soy Lecithin, Choline Chloride, Ascorbic Acid, Carrageenan, Zinc Sulfate, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Gellan Gum, Niacinamide, Calcium Pantothenate, Manganese Sulfate, Cupric Sulfate, Vitamin A Palmitate, Thiamine Chloride Hydrochloride, Pyridoxine Hydrochloride,

Riboflavin, Folic Acid, Sodium Molybdate, Chromium Chloride, Biotin, Potassium Iodide, Sodium Selenate, Phylloquinone, Vitamin D₃ and Cyanocobalamin.

Protein:

- 5 The protein source is a blend of two high-biologic-value proteins: casein and soy.

Sodium and calcium caseinates	85%
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Soy protein isolate	15%
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Fat:

- 10 The fat source is a blend of three oils: high-oleic safflower, canola, and soy.

High-oleic safflower oil	40%
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Canola oil	30%
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Soy oil	30%
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- 15 The level of fat in ENSURE HIGH PROTEIN meets American Heart Association (AHA) guidelines. The 6 grams of fat in ENSURE HIGH PROTEIN represent 24% of the total calories, with 2.6% of the fat being from saturated fatty acids and 7.9% from polyunsaturated fatty acids. These values are within the AHA guidelines of $\leq 30\%$ of total calories from fat, $< 10\%$ of the calories from saturated fatty acids, and $\leq 10\%$ of total calories from
- 20 polyunsaturated fatty acids.

Carbohydrate:

- ENSURE HIGH PROTEIN contains a combination of maltodextrin and sucrose. The mild sweetness and flavor variety (vanilla supreme, chocolate royal, wild berry, and banana), plus VARI-FLAVORSO® Flavor Pacs in pecan,
- 25 cherry, strawberry, lemon, and orange, help to prevent flavor fatigue and aid in patient compliance.

Vanilla and other nonchocolate flavors

Sucrose	60%
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Maltodextrin	40%
Chocolate	
Sucrose	70%
Maltodextrin	30%

5

D. ENSURE ® LIGHT

Usage: ENSURE LIGHT is a low-fat liquid food designed for use as an oral nutritional supplement with or between meals. ENSURE LIGHT is lactose- and gluten-free, and is suitable for use in modified diets, including low-cholesterol diets.

10

Patient Conditions:

- For normal-weight or overweight patients who need extra nutrition in a supplement that contains 50% less fat and 20% fewer calories than ENSURE
- For healthy adults who don't eat right and need extra nutrition

15

Features:

- Low in fat and saturated fat
- Contains 3 g of total fat per serving and < 5 mg cholesterol
- Rich, creamy taste
- Excellent source of calcium and other essential vitamins and minerals
- For low-cholesterol diets
- Lactose-free, easily digested

20

Ingredients:

French Vanilla: ®-D Water, Maltodextrin (Corn), Sugar (Sucrose), Calcium Caseinate, High-Oleic Safflower Oil, Canola Oil, Magnesium Chloride, Sodium Citrate, Potassium Citrate, Potassium Phosphate Dibasic, Magnesium Phosphate Dibasic, Natural and Artificial Flavor, Calcium Phosphate Tribasic, Cellulose Gel, Choline Chloride, Soy Lecithin, Carrageenan, Salt (Sodium Chloride),

25

Ascorbic Acid, Cellulose Gum, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Zinc Sulfate, Niacinamide, Manganese Sulfate, Calcium Pantothenate, Cupric Sulfate, Thiamine Chloride Hydrochloride, Vitamin A Palmitate, Pyridoxine Hydrochloride, Riboflavin, Chromium Chloride, Folic Acid, Sodium
5 Molybdate, Biotin, Potassium Iodide, Sodium Selenate, Phylloquinone, Vitamin D₃ and Cyanocobalamin.

Protein:

The protein source is calcium caseinate.

Calcium caseinate	100%
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10 **Fat**

The fat source is a blend of two oils: high-oleic safflower and canola.

High-oleic safflower oil	70%
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Canola oil	30%
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15 The level of fat in ENSURE LIGHT meets American Heart Association (AHA) guidelines. The 3 grams of fat in ENSURE LIGHT represent 13.5% of the total calories, with 1.4% of the fat being from saturated fatty acids and 2.6% from polyunsaturated fatty acids. These values are within the AHA guidelines of $\leq 30\%$ of total calories from fat, $< 10\%$ of the calories from saturated fatty acids, and $\leq 10\%$ of total calories from polyunsaturated fatty acids.

20 **Carbohydrate**

ENSURE LIGHT contains a combination of maltodextrin and sucrose. The chocolate flavor contains corn syrup as well. The mild sweetness and flavor variety (French vanilla, chocolate supreme, strawberry swirl), plus VARI-FLAVORS® Flavor Pacs in pecan, cherry, strawberry, lemon, and
25 orange, help to prevent flavor fatigue and aid in patient compliance.

Vanilla and other nonchocolate flavors

Sucrose	51%
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Maltodextrin	49%
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Chocolate

Sucrose	47.0%
Corn Syrup	26.5%
Maltodextrin	26.5%

5 **Vitamins and Minerals**

An 8-fl-oz serving of ENSURE LIGHT provides at least 25% of the RDIs for 24 key vitamins and minerals.

Caffeine

Chocolate flavor contains 2.1 mg caffeine/8 fl oz.

10

E. ENSURE PLUS®

Usage: ENSURE PLUS is a high-calorie, low-residue liquid food for use when extra calories and nutrients, but a normal concentration of protein, are needed. It is designed primarily as an oral nutritional supplement to be used with or between meals or, in appropriate amounts, as a meal replacement. ENSURE PLUS is lactose- and gluten-free. Although it is primarily an oral nutritional supplement, it can be fed by tube.

15

Patient Conditions:

- For patients who require extra calories and nutrients, but a normal concentration of protein, in a limited volume
- For patients who need to gain or maintain healthy weight

20

Features

- Rich, creamy taste
- Good source of essential vitamins and minerals

25

Ingredients

Vanilla: ®-D Water, Corn Syrup, Maltodextrin (Corn), Corn Oil, Sodium and Calcium Caseinates, Sugar (Sucrose), Soy Protein Isolate, Magnesium Chloride,

- Potassium Citrate, Calcium Phosphate Tribasic, Soy Lecithin, Natural and Artificial Flavor, Sodium Citrate, Potassium Chloride, Choline Chloride, Ascorbic Acid, Carrageenan, Zinc Sulfate, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Niacinamide, Calcium Pantothenate, Manganese Sulfate, Cupric Sulfate, Thiamine Chloride Hydrochloride, Pyridoxine Hydrochloride, Riboflavin, Vitamin A Palmitate, Folic Acid, Biotin, Chromium Chloride, Sodium Molybdate, Potassium Iodide, Sodium Selenite, Phylloquinone, Cyanocobalamin and Vitamin D₃.

Protein

- 10 The protein source is a blend of two high-biologic-value proteins: casein and soy.

Sodium and calcium caseinates	84%
Soy protein isolate	16%

Fat

- 15 The fat source is corn oil.

Corn oil	100%
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Carbohydrate

- 20 ENSURE PLUS contains a combination of maltodextrin and sucrose. The mild sweetness and flavor variety (vanilla, chocolate, strawberry, coffee, butter pecan, and eggnog), plus VARI-FLAVORS® Flavor Pacs in pecan, cherry, strawberry, lemon, and orange, help to prevent flavor fatigue and aid in patient compliance.

Vanilla, strawberry, butter pecan, and coffee flavors

Corn Syrup	39%
25 Maltodextrin	38%
Sucrose	23%

Chocolate and eggnog flavors

Corn Syrup	36%
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Maltodextrin	34%
Sucrose	30%

Vitamins and Minerals

5 An 8-fl-oz serving of ENSURE PLUS provides at least 15% of the RDIs for 25 key Vitamins and minerals.

Caffeine

Chocolate flavor contains 3.1 mg Caffeine/8 fl oz. Coffee flavor contains a trace amount of caffeine.

10 **F. ENSURE PLUS® HN**

Usage: ENSURE PLUS HN is a nutritionally complete high-calorie, high-nitrogen liquid food designed for people with higher calorie and protein needs or limited volume tolerance. It may be used for oral supplementation or for total nutritional support by tube. ENSURE PLUS HN is lactose- and gluten-free.

Patient Conditions:

- For patients with increased calorie and protein needs, such as following surgery or injury
- For patients with limited volume tolerance and early satiety

20 **Features**

- For supplemental or total nutrition
- For oral or tube feeding
- 1.5 CaV/mL
- High nitrogen
- 25 • Calorically dense

Ingredients

Vanilla: ©-D Water, Maltodextrin (Corn), Sodium and Calcium Caseinates,
Corn Oil, Sugar (Sucrose), Soy Protein Isolate, Magnesium Chloride, Potassium
Citrate, Calcium Phosphate Tribasic, Soy Lecithin, Natural and Artificial
5 Flavor, Sodium Citrate, Choline Chloride, Ascorbic Acid, Taurine, L-Carnitine,
Zinc Sulfate, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Niacinamide,
Carrageenan, Calcium Pantothenate, Manganese Sulfate, Cupric Sulfate,
Thiamine Chloride Hydrochloride, Pyridoxine Hydrochloride, Riboflavin,
Vitamin A Palmitate, Folic Acid, Biotin, Chromium Chloride, Sodium
10 Molybdate, Potassium Iodide, Sodium Selenite, Phylloquinone,
Cyanocobalamin and Vitamin D₃.

G. ENSURE® POWDER

Usage: ENSURE POWDER (reconstituted with water) is a low-residue
15 liquid food designed primarily as an oral nutritional supplement to be used with
or between meals. ENSURE POWDER is lactose- and gluten-free, and is
suitable for use in modified diets, including low-cholesterol diets.

Patient Conditions:

- For patients on modified diets
- 20 • For elderly patients at nutrition risk
- For patients recovering from illness/surgery
- For patients who need a low-residue diet

Features

- Convenient, easy to mix
- 25 • Low in saturated fat
- Contains 9 g of total fat and < 5 mg of cholesterol per serving
- High in vitamins and minerals
- For low-cholesterol diets

- Lactose-free, easily digested

Ingredients: @-D Corn Syrup, Maltodextrin (Corn), Sugar (Sucrose), Corn Oil, Sodium and Calcium Caseinates, Soy Protein Isolate, Artificial Flavor, Potassium Citrate, Magnesium Chloride, Sodium Citrate, Calcium Phosphate

5 Tribasic, Potassium Chloride, Soy Lecithin, Ascorbic Acid, Choline Chloride, Zinc Sulfate, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Niacinamide, Calcium Pantothenate, Manganese Sulfate, Thiamine Chloride Hydrochloride, Cupric Sulfate, Pyridoxine Hydrochloride, Riboflavin, Vitamin A Palmitate, Folic Acid, Biotin, Sodium Molybdate, Chromium Chloride, Potassium Iodide,

10 Sodium Selenate, Phylloquinone, Vitamin D₃ and Cyanocobalamin.

Protein

The protein source is a blend of two high-biologic-value proteins: casein and soy.

	Sodium and calcium caseinates	84%
15	Soy protein isolate	16%

Fat

The fat source is corn oil.

	Corn oil	100%
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Carbohydrate

20 ENSURE POWDER contains a combination of corn syrup, maltodextrin, and sucrose. The mild sweetness of ENSURE POWDER, plus VARI-FLAVORS® Flavor Pacs in pecan, cherry, strawberry, lemon, and orange, helps to prevent flavor fatigue and aid in patient compliance.

Vanilla

25	Corn Syrup	35%
	Maltodextrin	35%
	Sucrose	30%

H. ENSURE® PUDDING

Usage: ENSURE PUDDING is a nutrient-dense supplement providing balanced nutrition in a nonliquid form to be used with or between meals. It is appropriate for consistency-modified diets (e.g., soft, pureed, or full liquid) or for people with swallowing impairments. ENSURE PUDDING is gluten-free.

Patient Conditions:

- For patients on consistency-modified diets (e.g., soft, pureed, or full liquid)
- For patients with swallowing impairments

Features

- Rich and creamy, good taste
- Good source of essential vitamins and minerals Convenient-needs no refrigeration
- Gluten-free

Nutrient Profile per 5 oz: Calories 250, Protein 10.9%, Total Fat 34.9%, Carbohydrate 54.2%

Ingredients:

Vanilla: ©-D Nonfat Milk, Water, Sugar (Sucrose), Partially Hydrogenated Soybean Oil, Modified Food Starch, Magnesium Sulfate, Sodium Stearoyl Lactylate, Sodium Phosphate Dibasic, Artificial Flavor, Ascorbic Acid, Zinc Sulfate, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Choline Chloride, Niacinamide, Manganese Sulfate, Calcium Pantothenate, FD&C Yellow #5, Potassium Citrate, Cupric Sulfate, Vitamin A Palmitate, Thiamine Chloride Hydrochloride, Pyridoxine Hydrochloride, Riboflavin, FD&C Yellow #6, Folic Acid, Biotin, Phylloquinone, Vitamin D3 and Cyanocobalamin.

Protein

The protein source is nonfat milk.

Nonfat milk

100%

Fat

The fat source is hydrogenated soybean oil.

Hydrogenated soybean oil	100%
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Carbohydrate

5 ENSURE PUDDING contains a combination of sucrose and modified food starch. The mild sweetness and flavor variety (vanilla, chocolate, butterscotch, and tapioca) help prevent flavor fatigue. The product contains 9.2 grams of lactose per serving.

Vanilla and other nonchocolate flavors

10	Sucrose	56%
	Lactose	27%
	Modified food starch	17%

Chocolate

	Sucrose	58%
15	Lactose	26%
	Modified food starch	16%

I. ENSURE® WITH FIBER

20 Usage: ENSURE WITH FIBER is a fiber-containing, nutritionally complete liquid food designed for people who can benefit from increased dietary fiber and nutrients. ENSURE WITH FIBER is suitable for people who do not require a low-residue diet. It can be fed orally or by tube, and can be used as a nutritional supplement to a regular diet or, in appropriate amounts, as a meal replacement. ENSURE WITH FIBER is lactose- and gluten-free, and is

25 suitable for use in modified diets, including low-cholesterol diets.

Patient Conditions

- For patients who can benefit from increased dietary fiber and nutrients

Features

- New advanced formula-low in saturated fat, higher in vitamins and minerals
- Contains 6 g of total fat and < 5 mg of cholesterol per serving
- Rich, creamy taste
- 5 • Good source of fiber
- Excellent source of essential vitamins and minerals
- For low-cholesterol diets
- Lactose- and gluten-free

Ingredients

- 10 **Vanilla:** ®-D Water, Maltodextrin (Corn), Sugar (Sucrose), Sodium and Calcium Caseinates, Oat Fiber, High-Oleic Safflower Oil, Canola Oil, Soy Protein Isolate, Corn Oil, Soy Fiber, Calcium Phosphate Tribasic, Magnesium Chloride, Potassium Citrate, Cellulose Gel, Soy Lecithin, Potassium Phosphate Dibasic, Sodium Citrate, Natural and Artificial Flavors, Choline Chloride,
- 15 Magnesium Phosphate, Ascorbic Acid, Cellulose Gum, Potassium Chloride, Carrageenan, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Zinc Sulfate, Niacinamide, Manganese Sulfate, Calcium Pantothenate, Cupric Sulfate, Vitamin A Palmitate, Thiamine Chloride Hydrochloride, Pyridoxine Hydrochloride, Riboflavin, Folic Acid, Chromium Chloride, Biotin, Sodium
- 20 Molybdate, Potassium Iodide, Sodium Selenate, Phylloquinone, Vitamin D₃ and Cyanocobalamin.

Protein

The protein source is a blend of two high-biologic-value proteins- casein and soy.

25	Sodium and calcium caseinates	80%
	Soy protein isolate	20%

Fat

The fat source is a blend of three oils: high-oleic safflower, canola, and corn.

	High-oleic safflower oil	40%
5	Canola oil	40%
	Corn oil	20%

The level of fat in ENSURE WITH FIBER meets American Heart Association (AHA) guidelines. The 6 grams of fat in ENSURE WITH FIBER represent 22% of the total calories, with 2.01 % of the fat being from saturated fatty acids and 6.7% from polyunsaturated fatty acids. These values are within the AHA guidelines of $\leq 30\%$ of total calories from fat, $< 10\%$ of the calories from saturated fatty acids, and $\leq 10\%$ of total calories from polyunsaturated fatty acids.

Carbohydrate

ENSURE WITH FIBER contains a combination of maltodextrin and sucrose. The mild sweetness and flavor variety (vanilla, chocolate, and butter pecan), plus VARI-FLAVORS® Flavor Pacs in pecan, cherry, strawberry, lemon, and orange, help to prevent flavor fatigue and aid in patient compliance.

Vanilla and other nonchocolate flavors

20	Maltodextrin	66%
	Sucrose	25%
	Oat Fiber	7%
	Soy Fiber	2%

Chocolate

25	Maltodextrin	55%
	Sucrose	36%
	Oat Fiber	7%

Soy Fiber

2%

Fiber

The fiber blend used in ENSURE WITH FIBER consists of oat fiber and soy polysaccharide. This blend results in approximately 4 grams of total dietary fiber per 8-fl-oz can. The ratio of insoluble to soluble fiber is 95:5.

The various nutritional supplements described above and known to others of skill in the art can be substituted and/or supplemented with the PUFAs of this invention.

J. Oxepa™ Nutritional Product

Oxepa is low-carbohydrate, calorically dense enteral nutritional product designed for the dietary management of patients with or at risk for ARDS. It has a unique combination of ingredients, including a patented oil blend containing eicosapentaenoic acid (EPA from fish oil), γ -linolenic acid (GLA from borage oil), and elevated antioxidant levels.

Caloric Distribution:

- Caloric density is high at 1.5 Cal/mL (355 Cal/8 fl oz), to minimize the volume required to meet energy needs.
- The distribution of Calories in Oxepa is shown in Table 7.

Table 7. Caloric Distribution of Oxepa			
	per 8 fl oz.	per liter	% of Cal
Calories	355	1,500	---
Fat (g)	22.2	93.7	55.2
Carbohydrate (g)	25	105.5	28.1
Protein (g)	14.8	62.5	16.7
Water (g)	186	785	---

Fat:

- Oxepa contains 22.2 g of fat per 8-fl oz serving (93.7 g/L).
- The fat source is a oil blend of 31.8% canola oil, 25% medium-chain triglycerides (MCTs), 20% borage oil, 20% fish oil, and 3.2 % soy lecithin. The typical fatty acid profile of Oxepa is shown in Table 8.

- Oxepa provides a balanced amount of polyunsaturated, monounsaturated, and saturated fatty acids, as shown in Table 10.
- Medium-chain triglycerides (MCTs) -- 25% of the fat blend -- aid gastric emptying because they are absorbed by the intestinal tract without emulsification by bile acids.

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The various fatty acid components of Oxepa™ nutritional product can be substituted and/or supplemented with the PUFAs of this invention.

Table 8. Typical Fatty Acid Profile			
	% Total Fatty Acids	g/8 fl oz*	g/L*
Caproic (6:0)	0.2	0.04	0.18
Caprylic (8:0)	14.69	3.1	13.07
Capric (10:0)	11.06	2.33	9.87
Palmitic (16:0)	5.59	1.18	4.98
Palmitoleic (16:1n-7)	1.82	0.38	1.62
Stearic (18:0)	1.84	0.39	1.64
Oleic (18:1n-9)	24.44	5.16	21.75
Linoleic (18:2n-6)	16.28	3.44	14.49
α -Linolenic (18:3n-3)	3.47	0.73	3.09
γ -Linolenic (18:3n-6)	4.82	1.02	4.29
Eicosapentaenoic (20:5n-3)	5.11	1.08	4.55
n-3-Docosapentaenoic (22:5n-3)	0.55	0.12	0.49
Docosahexaenoic (22:6n-3)	2.27	0.48	2.02
Others	7.55	1.52	6.72

* Fatty acids equal approximately 95% of total fat.

Table 9. Fat Profile of Oxepa.	
% of total calories from fat	55.2
Polyunsaturated fatty acids	31.44 g/L
Monounsaturated fatty acids	25.53 g/L
Saturated fatty acids	32.38 g/L
n-6 to n-3 ratio	1.75:1
Cholesterol	9.49 mg/8 fl oz 40.1 mg/L

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Carbohydrate:

- The carbohydrate content is 25.0 g per 8-fl-oz serving (105.5 g/L).
- The carbohydrate sources are 45% maltodextrin (a complex carbohydrate) and 55% sucrose (a simple sugar), both of which are readily digested and absorbed.

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- The high-fat and low-carbohydrate content of Oxepa is designed to minimize carbon dioxide (CO₂) production. High CO₂ levels can complicate weaning in ventilator-dependent patients. The low level of carbohydrate also may be useful for those patients who have developed stress-induced hyperglycemia.

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- Oxepa is lactose-free.

Dietary carbohydrate, the amino acids from protein, and the glycerol moiety of fats can be converted to glucose within the body. Throughout this process, the carbohydrate requirements of glucose-dependent tissues (such as the central nervous system and red blood cells) are met. However, a diet free of carbohydrates can lead to ketosis, excessive catabolism of tissue protein, and loss of fluid and electrolytes. These effects can be prevented by daily ingestion of 50 to 100 g of digestible carbohydrate, if caloric intake is adequate. The carbohydrate level in Oxepa is also sufficient to minimize gluconeogenesis, if energy needs are being met.

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Protein:

- Oxepa contains 14.8 g of protein per 8-fl-oz serving (62.5 g/L).
- The total calorie/nitrogen ratio (150:1) meets the need of stressed patients.
- Oxepa provides enough protein to promote anabolism and the maintenance of lean body mass without precipitating respiratory problems. High protein intakes are a concern in patients with respiratory insufficiency. Although protein has little effect on CO₂ production, a high protein diet will increase ventilatory drive.

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- The protein sources of Oxepa are 86.8% sodium caseinate and 13.2% calcium caseinate.
- As demonstrated in Table 11, the amino acid profile of the protein system in Oxepa meets or surpasses the standard for high quality protein set by theNational Academy of Sciences.
- Oxepa is gluten-free.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

SEQUENCE LISTING

5

(1) GENERAL INFORMATION:

- 10 (i) APPLICANT: KNUTZON, DEBORAH
MURKERJI, PRADIP
HUANG, YUNG-SHENG
THURMOND, JENNIFER
CHAUDHARY, SUNITA
LEONARD, AMANDA
- 15 (ii) TITLE OF INVENTION: METHODS AND COMPOSITIONS FOR SYNTHESIS
OF LONG CHAIN POLY-UNSATURATED FATTY ACIDS IN PLANTS
- (iii) NUMBER OF SEQUENCES: 52
- 20 (iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: LIMBACH & LIMBACH L.L.P.
(B) STREET: 2001 FERRY BUILDING
(C) CITY: SAN FRANCISCO
(D) STATE: CA
25 (E) COUNTRY: USA
(F) ZIP: 94111
- (v) COMPUTER READABLE FORM:
30 (A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: Microsoft Word
- 35 (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:
- 40 (vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/834,033
(B) FILING DATE: 11-APR-1997
- (vii) PRIOR APPLICATION DATA:
45 (A) APPLICATION NUMBER: US 08/833,610
(B) FILING DATE: 11-APR-1997
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55 (A) TELEPHONE: (415) 433-4150
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(C) TELEX: N/A

(2) INFORMATION FOR SEQ ID NO:1:

60

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1617 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: DNA (genomic)

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

	CGACACTCCT TCCTTCTTCT CACCCGTCCT AGTCCCCTTC AACCCCCCTC TTTGACAAAG	60
	ACAACAAACC ATGGCTGCTG CTCCCAGTGT GAGGACGTTT ACTCGGGCCG AGGTTTGTAA	120
15	TGCCGAGGCT CTGAATGAGG GCAAGAAGGA TGCCGAGGCA CCCTTCTTGA TGATCATCGA	180
	CAACAAGGTG TACGATGTCC GCGAGTTCGT CCCTGATCAT CCCGGTGGAA GTGTGATTCT	240
20	CACGCACGTT GGCAAGGACG GCACTGACGT CTTTGACACT TTTCACCCCG AGGCTGCTTG	300
	GGAGACTCTT GCCAACTTTT ACGTTGGTGA TATTGACGAG AGCGACCGCG ATATCAAGAA	360
	TGATGACTTT GCGGCCGAGG TCCGCAAGCT GCGTACCTTG TTCCAGTCTC TTGGTTACTA	420
25	CGATTCTTCC AAGGCATACT ACGCCTTCAA GGTCTCGTTC AACCTCTGCA TCTGGGGTTT	480
	GTCGACGGTC ATTGTGGCCA AGTGGGGCCA GACCTCGACC CTCGCCAACG TGCTCTCGGC	540
30	TGCGCTTTTG GGTCTGTTCT GGCAGCAGTG CGGATGGTTG GCTCACGACT TTTTGCATCA	600
	CCAGGTCTTC CAGGACCGTT TCTGGGGTGA TCTTTTCGGC GCCTTCTTGG GAGGTGTCTG	660
	CCAGGGCTTC TCGTCCTCGT GGTGGAAGGA CAAGCACAAC ACTCACCACG CCGCCCCCAA	720
35	CGTCCACGGC GAGGATCCCG ACATTGACAC CCACCCTCTG TTGACCTGGA GTGAGCATGC	780
	GTTGGAGATG TTCTCGGATG TCCCAGATGA GGAGCTGACC CGCATGTGGT CGCGTTTCAT	840
40	GGTCCTGAAC CAGACCTGGT TTTACTTCCC CATTCTCTCG TTTGCCCGTC TCTCCTGGTG	900
	CCTCCAGTCC ATTCTCTTTG TGCTGCCTAA CGGTCAGGCC CACAAGCCCT CGGGCGCGCG	960
	TGTGCCCATC TCGTTGGTCG AGCAGCTGTC GCTTGCGATG CACTGGACCT GGTACCTCGC	1020
45	CACCATGTTC CTGTTTCATCA AGGATCCCGT CAACATGCTG GTGTACTTTT TGGTGTGCGA	1080
	GGCGGTGTGC GGAAACTTGT TGGCGATCGT GTTCTCGCTC AACCACAACG GTATGCCTGT	1140
50	GATCTCGAAG GAGGAGGCGG TCGATATGGA TTTCTTCACG AAGCAGATCA TCACGGGTCG	1200
	TGATGTCCAC CCGGGTCTAT TTGCCAACTG GTTCACGGGT GGATTGAACT ATCAGATCGA	1260
	GCACCACTTG TTCCCTTCGA TGCCTCGCCA CAACTTTTCA AAGATCCAGC CTGCTGTCGA	1320
55	GACCCTGTGC AAAAAGTACA ATGTCCGATA CCACACCACC GGTATGATCG AGGGAAGTGC	1380
	AGAGGTCTTT AGCCGTCTGA ACGAGGTCTC CAAGGCTGCC TCCAAGATGG GTAAGGCGCA	1440
60	GTAAAAAAA AAACAAGGAC GTTTTTTTTT GCCAGTGCCT GTGCCTGTGC CTGCTTCCCT	1500
	TGTCAAGTCG AGCGTTTCTG GAAAGGATCG TTCAGTGCAG TATCATCATT CTCCTTTTAC	1560

CCCCCGCTCA TATCTCATTC ATTTCTCTTA TTAAACAAC TGTTCACCCG 1617

5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

10

- (A) LENGTH: 457 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: peptide

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

25

Met Ala Ala Ala Pro Ser Val Arg Thr Phe Thr Arg Ala Glu Val Leu
1 5 10 15

Asn Ala Glu Ala Leu Asn Glu Gly Lys Lys Asp Ala Glu Ala Pro Phe
20 25 30

Leu Met Ile Ile Asp Asn Lys Val Tyr Asp Val Arg Glu Phe Val Pro
35 40 45

30

Asp His Pro Gly Gly Ser Val Ile Leu Thr His Val Gly Lys Asp Gly
50 55 60

35

Thr Asp Val Phe Asp Thr Phe His Pro Glu Ala Ala Trp Glu Thr Leu
65 70 75 80

Ala Asn Phe Tyr Val Gly Asp Ile Asp Glu Ser Asp Arg Asp Ile Lys
85 90 95

40

Asn Asp Asp Phe Ala Ala Glu Val Arg Lys Leu Arg Thr Leu Phe Gln
100 105 110

Ser Leu Gly Tyr Tyr Asp Ser Ser Lys Ala Tyr Tyr Ala Phe Lys Val
115 120 125

45

Ser Phe Asn Leu Cys Ile Trp Gly Leu Ser Thr Val Ile Val Ala Lys
130 135 140

50

Trp Gly Gln Thr Ser Thr Leu Ala Asn Val Leu Ser Ala Ala Leu Leu
145 150 155 160

Gly Leu Phe Trp Gln Gln Cys Gly Trp Leu Ala His Asp Phe Leu His
165 170 175

55

His Gln Val Phe Gln Asp Arg Phe Trp Gly Asp Leu Phe Gly Ala Phe
180 185 190

Leu Gly Gly Val Cys Gln Gly Phe Ser Ser Ser Trp Trp Lys Asp Lys
195 200 205

60

His Asn Thr His His Ala Ala Pro Asn Val His Gly Glu Asp Pro Asp
210 215 220

Ile Asp Thr His Pro Leu Leu Thr Trp Ser Glu His Ala Leu Glu Met
 225 230 235 240
 5 Phe Ser Asp Val Pro Asp Glu Glu Leu Thr Arg Met Trp Ser Arg Phe
 245 250 255
 Met Val Leu Asn Gln Thr Trp Phe Tyr Phe Pro Ile Leu Ser Phe Ala
 260 265 270
 10 Arg Leu Ser Trp Cys Leu Gln Ser Ile Leu Phe Val Leu Pro Asn Gly
 275 280 285
 Gln Ala His Lys Pro Ser Gly Ala Arg Val Pro Ile Ser Leu Val Glu
 290 295 300
 15 Gln Leu Ser Leu Ala Met His Trp Thr Trp Tyr Leu Ala Thr Met Phe
 305 310 315 320
 Leu Phe Ile Lys Asp Pro Val Asn Met Leu Val Tyr Phe Leu Val Ser
 325 330 335
 20 Gln Ala Val Cys Gly Asn Leu Leu Ala Ile Val Phe Ser Leu Asn His
 340 345 350
 Asn Gly Met Pro Val Ile Ser Lys Glu Glu Ala Val Asp Met Asp Phe
 355 360 365
 25 Phe Thr Lys Gln Ile Ile Thr Gly Arg Asp Val His Pro Gly Leu Phe
 370 375 380
 30 Ala Asn Trp Phe Thr Gly Gly Leu Asn Tyr Gln Ile Glu His His Leu
 385 390 395 400
 Phe Pro Ser Met Pro Arg His Asn Phe Ser Lys Ile Gln Pro Ala Val
 405 410 415
 35 Glu Thr Leu Cys Lys Lys Tyr Asn Val Arg Tyr His Thr Thr Gly Met
 420 425 430
 40 Ile Glu Gly Thr Ala Glu Val Phe Ser Arg Leu Asn Glu Val Ser Lys
 435 440 445
 Ala Ala Ser Lys Met Gly Lys Ala Gln
 450 455

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1488 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTCCCTGTC GCTGTCGGCA CACCCCATCC TCCCTCGCTC CCTCTGCGTT TGTCTTGGC 60

CCACCGTCTC TCCTCCACCC TCCGAGACGA CTGCAACTGT AATCAGGAAC CGACAAATAC 120
 ACGATTTCTT TTTACTCAGC ACCAACTCAA AATCCTCAAC CGCAACCCTT TTTCAGGATG 180
 5 GCACCTCCCA AACTATATCGA TGCCGGTTTG ACCCAGCGTC ATATCAGCAC CTCGGCCCCA 240
 AACTCGGCCA AGCCTGCCTT CGAGCGCAAC TACCAGCTCC CCGAGTTCAC CATCAAGGAG 300
 10 ATCCGAGAGT GCATCCCTGC CCACTGCTTT GAGCGCTCCG GTCTCCGTGG TCTCTGCCAC 360
 GTTGCCATCG ATCTGACTTG GGCCTCGCTC TTGTTCTGG CTGCGACCCA GATCGACAAG 420
 TTTGAGAATC CCTTGATCCG CTATTTGGCC TGGCCTGTTT ACTGGATCAT GCAGGGTATT 480
 15 GTCTGCACCG GTGTCTGGGT GCTGGCTCAC GAGTGTGGTC ATCAGTCCTT CTCGACCTCC 540
 AAGACCCTCA ACAACACAGT TGGTTGGATC TTGCACTCGA TGCTCTTGGT CCCCTACCAC 600
 20 TCCTGGAGAA TCTCGCACTC GAAGCACCAC AAGGCCACTG GCCATATGAC CAAGGACCAG 660
 GTCTTTGTGC CCAAGACCCG CTCCCAGGTT GGCTTGCCTC CCAAGGAGAA CGCTGCTGCT 720
 GCCGTTCAGG AGGAGGACAT GTCCGTGCAC CTGGATGAGG AGGCTCCCAT TGTGACTTTG 780
 25 TTCTGGATGG TGATCCAGTT CTTGTTCCGA TGGCCCGCGT ACCTGATTAT GAACGCCTCT 840
 GGCCAAGACT ACGGCCGCTG GACCTCGCAC TTCCACACGT ACTCGCCCAT CTTTGAGCCC 900
 30 CGCAACTTTT TCGACATTAT TATCTCGGAC CTCGGTGTGT TGGCTGCCCT CGGTGCCCTG 960
 ATCTATGCCT CCATGCAGTT GTCGCTCTTG ACCGTCACCA AGTACTATAT TGTCCCCTAC 1020
 CTCTTTGTCA ACTTTTGGTT GGTCTGATC ACCTTCTTGC AGCACACCGA TCCCAAGCTG 1080
 35 CCCCATTACC GCGAGGGTGC CTGGAATTC CAGCGTGGAG CTCTTTGCAC CGTTGACCGC 1140
 TCGTTTGGCA AGTTCTTGGA CCATATGTTT CACGGCATTG TCCACACCCA TGTGGCCCAT 1200
 40 CACTTGTTCT CGCAAATGCC GTTCTACCAT GCTGAGGAAG CTACCTATCA TCTCAAGAAA 1260
 CTGCTGGGAG AGTACTATGT GTACGACCCA TCCCGATCG TCGTTGCGGT CTGGAGGTCG 1320
 TTCCGTGAGT GCCGATTCGT GGAGGATCAG GGAGACGTGG TCTTTTCAA GAAGTAAAAA 1380
 45 AAAAGACAAT GGACCACACA CAACCTTGTC TCTACAGACC TACGTATCAT GTAGCCATAC 1440
 CACTTCATAA AAGAACATGA GCTCTAGAGG CGTGTCATTC GCGCCTCC 1488

50 (2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 399 amino acids
 (B) TYPE: amino acid
 55 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

60

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

	Met	Ala	Pro	Pro	Asn	Thr	Ile	Asp	Ala	Gly	Leu	Thr	Gln	Arg	His	Ile	
	1				5					10					15		
5	Ser	Thr	Ser	Ala	Pro	Asn	Ser	Ala	Lys	Pro	Ala	Phe	Glu	Arg	Asn	Tyr	
				20					25					30			
	Gln	Leu	Pro	Glu	Phe	Thr	Ile	Lys	Glu	Ile	Arg	Glu	Cys	Ile	Pro	Ala	
10			35					40					45				
	His	Cys	Phe	Glu	Arg	Ser	Gly	Leu	Arg	Gly	Leu	Cys	His	Val	Ala	Ile	
		50					55					60					
15	Asp	Leu	Thr	Trp	Ala	Ser	Leu	Leu	Phe	Leu	Ala	Ala	Thr	Gln	Ile	Asp	
	65					70					75					80	
	Lys	Phe	Glu	Asn	Pro	Leu	Ile	Arg	Tyr	Leu	Ala	Trp	Pro	Val	Tyr	Trp	
				85						90					95		
20	Ile	Met	Gln	Gly	Ile	Val	Cys	Thr	Gly	Val	Trp	Val	Leu	Ala	His	Glu	
				100					105					110			
	Cys	Gly	His	Gln	Ser	Phe	Ser	Thr	Ser	Lys	Thr	Leu	Asn	Asn	Thr	Val	
25			115					120					125				
	Gly	Trp	Ile	Leu	His	Ser	Met	Leu	Leu	Val	Pro	Tyr	His	Ser	Trp	Arg	
		130					135					140					
30	Ile	Ser	His	Ser	Lys	His	His	Lys	Ala	Thr	Gly	His	Met	Thr	Lys	Asp	
	145					150					155					160	
	Gln	Val	Phe	Val	Pro	Lys	Thr	Arg	Ser	Gln	Val	Gly	Leu	Pro	Pro	Lys	
					165					170					175		
35	Glu	Asn	Ala	Ala	Ala	Ala	Val	Gln	Glu	Glu	Asp	Met	Ser	Val	His	Leu	
				180					185					190			
	Asp	Glu	Glu	Ala	Pro	Ile	Val	Thr	Leu	Phe	Trp	Met	Val	Ile	Gln	Phe	
40			195					200					205				
	Leu	Phe	Gly	Trp	Pro	Ala	Tyr	Leu	Ile	Met	Asn	Ala	Ser	Gly	Gln	Asp	
		210					215					220					
45	Tyr	Gly	Arg	Trp	Thr	Ser	His	Phe	His	Thr	Tyr	Ser	Pro	Ile	Phe	Glu	
	225					230					235					240	
	Pro	Arg	Asn	Phe	Phe	Asp	Ile	Ile	Ile	Ser	Asp	Leu	Gly	Val	Leu	Ala	
					245					250					255		
50	Ala	Leu	Gly	Ala	Leu	Ile	Tyr	Ala	Ser	Met	Gln	Leu	Ser	Leu	Leu	Thr	
				260					265					270			
	Val	Thr	Lys	Tyr	Tyr	Ile	Val	Pro	Tyr	Leu	Phe	Val	Asn	Phe	Trp	Leu	
55			275					280					285				
	Val	Leu	Ile	Thr	Phe	Leu	Gln	His	Thr	Asp	Pro	Lys	Leu	Pro	His	Tyr	
		290					295					300					
60	Arg	Glu	Gly	Ala	Trp	Asn	Phe	Gln	Arg	Gly	Ala	Leu	Cys	Thr	Val	Asp	
	305					310					315					320	

5	Arg	Ser	Phe	Gly	Lys	Phe	Leu	Asp	His	Met	Phe	His	Gly	Ile	Val	His
					325					330					335	
	Thr	His	Val	Ala	His	His	Leu	Phe	Ser	Gln	Met	Pro	Phe	Tyr	His	Ala
				340					345					350		
10	Glu	Glu	Ala	Thr	Tyr	His	Leu	Lys	Lys	Leu	Leu	Gly	Glu	Tyr	Tyr	Val
			355					360					365			
	Tyr	Asp	Pro	Ser	Pro	Ile	Val	Val	Ala	Val	Trp	Arg	Ser	Phe	Arg	Glu
		370					375					380				
	Cys	Arg	Phe	Val	Glu	Asp	Gln	Gly	Asp	Val	Val	Phe	Phe	Lys	Lys	
	385					390					395					

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1483 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

	GCTTCCTCCA	GTTCATCCTC	CATTTTCGCCA	CCTGCATTCT	TTACGACCGT	TAAGCAAGAT	60
	GGGAACGGAC	CAAGGAAAAA	CCTTCACCTG	GGAAGAGCTG	GCGGCCCAT	ACACCAAGGA	120
35	CGACCTACTC	TTGGCCATCC	GCGGCAGGGT	GTACGATGTC	ACAAAGTTCT	TGAGCCGCCA	180
	TCCTGGTGGA	GTGGACACTC	TCCTGCTCGG	AGCTGGCCGA	GATGTTACTC	CGGTCTTTGA	240
40	GATGTATCAC	GCGTTTGGGG	CTGCAGATGC	CATTATGAAG	AAGTACTATG	TCGGTACACT	300
	GGTCTCGAAT	GAGCTGCCCA	TCTTCCCGGA	GCCAACGGTG	TTCCACAAA	CCATCAAGAC	360
	GAGAGTCGAG	GGCTACTTTA	CGGATCGGAA	CATTGATCCC	AAGAATAGAC	CAGAGATCTG	420
45	GGGACGATAC	GCTCTTATCT	TTGGATCCTT	GATCGCTTCC	TACTACGCGC	AGCTCTTTGT	480
	GCCTTTCGTT	GTCGAACGCA	CATGGCTTCA	GGTGGTGTTT	GCAATCATCA	TGGGATTTGC	540
50	GTGCGCACAA	GTCGGACTCA	ACCCTCTTCA	TGATGCGTCT	CACTTTTCAG	TGACCCACAA	600
	CCCCACTGTC	TGGAAGATTC	TGGGAGCCAC	GCACGACTTT	TTCAACGGAG	CATCGTACCT	660
	GGTGTGGATG	TACCAACATA	TGCTCGGCCA	TCACCCCTAC	ACCAACATTG	CTGGAGCAGA	720
55	TCCCACGCTG	TCGACGTCTG	AGCCCAGATG	TCGTCGTATC	AAGCCCAACC	AAAAGTGGTT	780
	TGTCAACCAC	ATCAACCAGC	ACATGTTTGT	TCCTTTCCTG	TACGGACTGC	TGGCGTTCAA	840
60	GGTGCGCATT	CAGGACATCA	ACATTTTGTA	CTTTGTCAAG	ACCAATGACG	CTATTCGTGT	900
	CAATCCCATC	TCGACATGGC	ACACTGTGAT	GTTCTGGGGC	GGCAAGGCTT	TCTTTGTCTG	960

GTATCGCCTG ATTGTTCCCC TGCAGTATCT GCCCCTGGGC AAGGTGCTGC TCTTGTTTAC 1020
 GGTCGCGGAC ATGGTGTCGT CTTACTGGCT GGCGCTGACC TTCCAGGCGA ACCACGTTGT 1080
 5 TGAGGAAGTT CAGTGGCCGT TGCCTGACGA GAACGGGATC ATCCAAAAGG ACTGGGCAGC 1140
 TATGCAGGTC GAGACTACGC AGGATTACGC ACACGATTCTG CACCTCTGGA CCAGCATCAC 1200
 10 TGGCAGCTTG AACTACCAGG CTGTGCACCA TCTGTTCCCC AACGTGTCGC AGCACCATTA 1260
 TCCCGATATT CTGGCCATCA TCAAGAACAC CTGCAGCGAG TACAAGGTTT CATACTTGT 1320
 CAAGGATACG TTTTGGCAAG CATTGCTTC ACATTGGAG CACTTGCGTG TTCTTGGACT 1380
 15 CCGTCCCAAG GAAGAGTAGA AGAAAAAAG CGCCGAATGA AGTATTGCCC CCTTTTCTC 1440
 CAAGAATGGC AAAAGGAGAT CAAGTGGACA TTCTCTATGA AGA 1483

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 446 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Gly Thr Asp Gln Gly Lys Thr Phe Thr Trp Glu Glu Leu Ala Ala
 1 5 10 15
 His Asn Thr Lys Asp Asp Leu Leu Leu Ala Ile Arg Gly Arg Val Tyr
 20 25 30
 40 Asp Val Thr Lys Phe Leu Ser Arg His Pro Gly Gly Val Asp Thr Leu
 35 40 45
 Leu Leu Gly Ala Gly Arg Asp Val Thr Pro Val Phe Glu Met Tyr His
 50 55 60
 45 Ala Phe Gly Ala Ala Asp Ala Ile Met Lys Lys Tyr Tyr Val Gly Thr
 65 70 75 80
 50 Leu Val Ser Asn Glu Leu Pro Ile Phe Pro Glu Pro Thr Val Phe His
 85 90 95
 Lys Thr Ile Lys Thr Arg Val Glu Gly Tyr Phe Thr Asp Arg Asn Ile
 100 105 110
 55 Asp Pro Lys Asn Arg Pro Glu Ile Trp Gly Arg Tyr Ala Leu Ile Phe
 115 120 125
 Gly Ser Leu Ile Ala Ser Tyr Tyr Ala Gln Leu Phe Val Pro Phe Val
 130 135 140
 60 Val Glu Arg Thr Trp Leu Gln Val Val Phe Ala Ile Ile Met Gly Phe
 145 150 155 160

	Ala	Cys	Ala	Gln	Val	Gly	Leu	Asn	Pro	Leu	His	Asp	Ala	Ser	His	Phe
					165					170					175	
5	Ser	Val	Thr	His	Asn	Pro	Thr	Val	Trp	Lys	Ile	Leu	Gly	Ala	Thr	His
				180					185					190		
10	Asp	Phe	Phe	Asn	Gly	Ala	Ser	Tyr	Leu	Val	Trp	Met	Tyr	Gln	His	Met
			195					200					205			
	Leu	Gly	His	His	Pro	Tyr	Thr	Asn	Ile	Ala	Gly	Ala	Asp	Pro	Asp	Val
		210					215					220				
15	Ser	Thr	Ser	Glu	Pro	Asp	Val	Arg	Arg	Ile	Lys	Pro	Asn	Gln	Lys	Trp
	225					230					235					240
	Phe	Val	Asn	His	Ile	Asn	Gln	His	Met	Phe	Val	Pro	Phe	Leu	Tyr	Gly
					245					250					255	
20	Leu	Leu	Ala	Phe	Lys	Val	Arg	Ile	Gln	Asp	Ile	Asn	Ile	Leu	Tyr	Phe
				260					265					270		
	Val	Lys	Thr	Asn	Asp	Ala	Ile	Arg	Val	Asn	Pro	Ile	Ser	Thr	Trp	His
25			275					280					285			
	Thr	Val	Met	Phe	Trp	Gly	Gly	Lys	Ala	Phe	Phe	Val	Trp	Tyr	Arg	Leu
		290					295					300				
30	Ile	Val	Pro	Leu	Gln	Tyr	Leu	Pro	Leu	Gly	Lys	Val	Leu	Leu	Leu	Phe
	305					310					315					320
	Thr	Val	Ala	Asp	Met	Val	Ser	Ser	Tyr	Trp	Leu	Ala	Leu	Thr	Phe	Gln
					325					330					335	
35	Ala	Asn	His	Val	Val	Glu	Glu	Val	Gln	Trp	Pro	Leu	Pro	Asp	Glu	Asn
				340					345					350		
	Gly	Ile	Ile	Gln	Lys	Asp	Trp	Ala	Ala	Met	Gln	Val	Glu	Thr	Thr	Gln
40			355					360					365			
	Asp	Tyr	Ala	His	Asp	Ser	His	Leu	Trp	Thr	Ser	Ile	Thr	Gly	Ser	Leu
		370					375					380				
45	Asn	Tyr	Gln	Ala	Val	His	His	Leu	Phe	Pro	Asn	Val	Ser	Gln	His	His
	385					390					395					400
	Tyr	Pro	Asp	Ile	Leu	Ala	Ile	Ile	Lys	Asn	Thr	Cys	Ser	Glu	Tyr	Lys
					405					410					415	
50	Val	Pro	Tyr	Leu	Val	Lys	Asp	Thr	Phe	Trp	Gln	Ala	Phe	Ala	Ser	His
				420					425					430		
	Leu	Glu	His	Leu	Arg	Val	Leu	Gly	Leu	Arg	Pro	Lys	Glu	Glu		
			435					440					445			

(2) INFORMATION FOR SEO ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 355 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

10	Glu	Val	Arg	Lys	Leu	Arg	Thr	Leu	Phe	Gln	Ser	Leu	Gly	Tyr	Tyr	Asp	
	1				5					10						15	
	Ser	Ser	Lys	Ala	Tyr	Tyr	Ala	Phe	Lys	Val	Ser	Phe	Asn	Leu	Cys	Ile	
				20					25					30			
15	Trp	Gly	Leu	Ser	Thr	Val	Ile	Val	Ala	Lys	Trp	Gly	Gln	Thr	Ser	Thr	
			35					40					45				
	Leu	Ala	Asn	Val	Leu	Ser	Ala	Ala	Leu	Leu	Gly	Leu	Phe	Trp	Gln	Gln	
		50					55					60					
20	Cys	Gly	Trp	Leu	Ala	His	Asp	Phe	Leu	His	His	Gln	Val	Phe	Gln	Asp	
	65					70					75					80	
	Arg	Phe	Trp	Gly	Asp	Leu	Phe	Gly	Ala	Phe	Leu	Gly	Gly	Val	Cys	Gln	
25					85					90					95		
	Gly	Phe	Ser	Ser	Ser	Trp	Trp	Lys	Asp	Lys	His	Asn	Thr	His	His	Ala	
				100					105					110			
30	Ala	Pro	Asn	Val	His	Gly	Glu	Asp	Pro	Asp	Ile	Asp	Thr	His	Pro	Leu	
			115					120					125				
	Leu	Thr	Trp	Ser	Glu	His	Ala	Leu	Glu	Met	Phe	Ser	Asp	Val	Pro	Asp	
35			130				135					140					
	Glu	Glu	Leu	Thr	Arg	Met	Trp	Ser	Arg	Phe	Met	Val	Leu	Asn	Gln	Thr	
	145					150					155					160	
	Trp	Phe	Tyr	Phe	Pro	Ile	Leu	Ser	Phe	Ala	Arg	Leu	Ser	Trp	Cys	Leu	
40					165					170					175		
	Gln	Ser	Ile	Leu	Phe	Val	Leu	Pro	Asn	Gly	Gln	Ala	His	Lys	Pro	Ser	
				180					185					190			
45	Gly	Ala	Arg	Val	Pro	Ile	Ser	Leu	Val	Glu	Gln	Leu	Ser	Leu	Ala	Met	
			195					200					205				
	His	Trp	Thr	Trp	Tyr	Leu	Ala	Thr	Met	Phe	Leu	Phe	Ile	Lys	Asp	Pro	
50		210					215					220					
	Val	Asn	Met	Leu	Val	Tyr	Phe	Leu	Val	Ser	Gln	Ala	Val	Cys	Gly	Asn	
	225					230					235					240	
	Leu	Leu	Ala	Ile	Val	Phe	Ser	Leu	Asn	His	Asn	Gly	Met	Pro	Val	Ile	
55				245						250					255		
	Ser	Lys	Glu	Glu	Ala	Val	Asp	Met	Asp	Phe	Phe	Thr	Lys	Gln	Ile	Ile	
				260					265					270			
60	Thr	Gly	Arg	Asp	Val	His	Pro	Gly	Leu	Phe	Ala	Asn	Trp	Phe	Thr	Gly	
			275					280					285				

Gly Leu Asn Tyr Gln Ile Glu His His Leu Phe Pro Ser Met Pro Arg
 290 295 300
 5 His Asn Phe Ser Lys Ile Gln Pro Ala Val Glu Thr Leu Cys Lys Lys
 305 310 315 320
 Tyr Asn Val Arg Tyr His Thr Thr Gly Met Ile Glu Gly Thr Ala Glu
 325 330 335
 10 Val Phe Ser Arg Leu Asn Glu Val Ser Lys Ala Ala Ser Lys Met Gly
 340 345 350
 Lys Ala Gln
 355
 15

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 104 amino acids
 20 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Val Thr Leu Tyr Thr Leu Ala Phe Val Ala Ala Asn Ser Leu Gly Val
 1 5 10 15
 35 Leu Tyr Gly Val Leu Ala Cys Pro Ser Val Xaa Pro His Gln Ile Ala
 20 25 30
 Ala Gly Leu Leu Gly Leu Leu Trp Ile Gln Ser Ala Tyr Ile Gly Xaa
 35 40 45
 40 Asp Ser Gly His Tyr Val Ile Met Ser Asn Lys Ser Asn Asn Xaa Phe
 50 55 60
 45 Ala Gln Leu Leu Ser Gly Asn Cys Leu Thr Gly Ile Ile Ala Trp Trp
 65 70 75 80
 Lys Trp Thr His Asn Ala His His Leu Ala Cys Asn Ser Leu Asp Tyr
 85 90 95
 50 Gly Pro Asn Leu Gln His Ile Pro
 100

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 252 amino acids
 55 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

5 Gly Val Leu Tyr Gly Val Leu Ala Cys Thr Ser Val Phe Ala His Gln
 1 5 10 15
 Ile Ala Ala Ala Leu Leu Gly Leu Leu Trp Ile Gln Ser Ala Tyr Ile
 20 25 30
 10 Gly His Asp Ser Gly His Tyr Val Ile Met Ser Asn Lys Ser Tyr Asn
 35 40 45
 15 Arg Phe Ala Gln Leu Leu Ser Gly Asn Cys Leu Thr Gly Ile Ser Ile
 50 55 60
 Ala Trp Trp Lys Trp Thr His Asn Ala His His Leu Ala Cys Asn Ser
 65 70 75 80
 20 Leu Asp Tyr Asp Pro Asp Leu Gln His Ile Pro Val Phe Ala Val Ser
 85 90 95
 Thr Lys Phe Phe Ser Ser Leu Thr Ser Arg Phe Tyr Asp Arg Lys Leu
 100 105 110
 25 Thr Phe Gly Pro Val Ala Arg Phe Leu Val Ser Tyr Gln His Phe Thr
 115 120 125
 30 Tyr Tyr Pro Val Asn Cys Phe Gly Arg Ile Asn Leu Phe Ile Gln Thr
 130 135 140
 Phe Leu Leu Leu Phe Ser Lys Arg Glu Val Pro Asp Arg Ala Leu Asn
 145 150 155 160
 35 Phe Ala Gly Ile Leu Val Phe Trp Thr Trp Phe Pro Leu Leu Val Ser
 165 170 175
 Cys Leu Pro Asn Trp Pro Glu Arg Phe Phe Phe Val Phe Thr Ser Phe
 180 185 190
 40 Thr Val Thr Ala Leu Gln His Ile Gln Phe Thr Leu Asn His Phe Ala
 195 200 205
 45 Ala Asp Val Tyr Val Gly Pro Pro Thr Gly Ser Asp Trp Phe Glu Lys
 210 215 220
 Gln Ala Ala Gly Thr Ile Asp Ile Ser Cys Arg Ser Tyr Met Asp Trp
 225 230 235 240
 50 Phe Phe Gly Gly Leu Gln Phe Gln Leu Glu His His
 245 250

(2) INFORMATION FOR SEQ ID NO:10:

- 55 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 125 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear
 60 (ii) MOLECULE TYPE: peptide

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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Gly Xaa Xaa Asn Phe Ala Gly Ile Leu Val Phe Trp Thr Trp Phe Pro
 1 5 10 15
 Leu Leu Val Ser Cys Leu Pro Asn Trp Pro Glu Arg Phe Xaa Phe Val
 20 25 30
 Phe Thr Gly Phe Thr Val Thr Ala Leu Gln His Ile Gln Phe Thr Leu
 35 40 45
 Asn His Phe Ala Ala Asp Val Tyr Val Gly Pro Pro Thr Gly Ser Asp
 50 55 60
 Trp Phe Glu Lys Gln Ala Ala Gly Thr Ile Asp Ile Ser Cys Arg Ser
 65 70 75 80
 Tyr Met Asp Trp Phe Phe Cys Gly Leu Gln Phe Gln Leu Glu His His
 85 90 95
 Leu Phe Pro Arg Leu Pro Arg Cys His Leu Arg Lys Val Ser Pro Val
 100 105 110
 Gly Gln Arg Gly Phe Gln Arg Lys Xaa Asn Leu Ser Xaa
 115 120 125

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 131 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: not relevant

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Pro Ala Thr Glu Val Gly Gly Leu Ala Trp Met Ile Thr Phe Tyr Val
 1 5 10 15
 Arg Phe Phe Leu Thr Tyr Val Pro Leu Leu Gly Leu Lys Ala Phe Leu
 20 25 30
 Gly Leu Phe Phe Ile Val Arg Phe Leu Glu Ser Asn Trp Phe Val Trp
 35 40 45
 Val Thr Gln Met Asn His Ile Pro Met His Ile Asp His Asp Arg Asn
 50 55 60
 Met Asp Trp Val Ser Thr Gln Leu Gln Ala Thr Cys Asn Val His Lys
 65 70 75 80
 Ser Ala Phe Asn Asp Trp Phe Ser Gly His Leu Asn Phe Gln Ile Glu
 85 90 95

His His Leu Phe Pro Thr Met Pro Arg His Asn Tyr His Xaa Val Ala
 100 105 110

5 Pro Leu Val Gln Ser Leu Cys Ala Lys His Gly Ile Glu Tyr Gln Ser
 115 120 125

Lys Pro Leu
 130

10 (2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- 15 (A) LENGTH: 87 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

25 Cys Ser Pro Lys Ser Ser Pro Thr Arg Asn Met Thr Pro Ser Pro Phe
 1 5 10 15

Ile Asp Trp Leu Trp Gly Gly Leu Asn Tyr Gln Ile Glu His His Leu
 20 25 30

30 Phe Pro Thr Met Pro Arg Cys Asn Leu Asn Arg Cys Met Lys Tyr Val
 35 40 45

35 Lys Glu Trp Cys Ala Glu Asn Asn Leu Pro Tyr Leu Val Asp Asp Tyr
 50 55 60

Phe Val Gly Tyr Asn Leu Asn Leu Gln Gln Leu Lys Asn Met Ala Glu
 65 70 75 80

40 Leu Val Gln Ala Lys Ala Ala
 85

(2) INFORMATION FOR SEQ ID NO:13:

45 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 143 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 50 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Arg His Glu Ala Ala Arg Gly Gly Thr Arg Leu Ala Tyr Met Leu Val
 1 5 10 15

60 Cys Met Gln Trp Thr Asp Leu Leu Trp Ala Ala Ser Phe Tyr Ser Arg
 20 25 30

	Phe	Phe	Leu	Ser	Tyr	Ser	Pro	Phe	Tyr	Gly	Ala	Thr	Gly	Thr	Leu	Leu
			35					40					45			
5	Leu	Phe	Val	Ala	Val	Arg	Val	Leu	Glu	Ser	His	Trp	Phe	Val	Trp	Ile
		50					55					60				
10	Thr	Gln	Met	Asn	His	Ile	Pro	Lys	Glu	Ile	Gly	His	Glu	Lys	His	Arg
	65					70					75					80
	Asp	Trp	Ala	Ser	Ser	Gln	Leu	Ala	Ala	Thr	Cys	Asn	Val	Glu	Pro	Ser
					85					90					95	
15	Leu	Phe	Ile	Asp	Trp	Phe	Ser	Gly	His	Leu	Asn	Phe	Gln	Ile	Glu	His
				100					105					110		
	His	Leu	Phe	Pro	Thr	Met	Thr	Arg	His	Asn	Tyr	Arg	Xaa	Val	Ala	Pro
			115					120					125			
20	Leu	Val	Lys	Ala	Phe	Cys	Ala	Lys	His	Gly	Leu	His	Tyr	Glu	Val	
		130					135					140				

(2) INFORMATION FOR SEQ ID NO:14:

25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 186 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

40	Leu	His	His	Thr	Tyr	Thr	Asn	Ile	Ala	Gly	Ala	Asp	Pro	Asp	Val	Ser
	1				5					10					15	
45	Thr	Ser	Glu	Pro	Asp	Val	Arg	Arg	Ile	Lys	Pro	Asn	Gln	Lys	Trp	Phe
				20					25					30		
50	Val	Asn	His	Ile	Asn	Gln	His	Met	Phe	Val	Pro	Phe	Leu	Tyr	Gly	Leu
			35					40					45			
55	Leu	Ala	Phe	Lys	Val	Arg	Ile	Gln	Asp	Ile	Asn	Ile	Leu	Tyr	Phe	Val
		50					55					60				
60	Lys	Thr	Asn	Asp	Ala	Ile	Arg	Val	Asn	Pro	Ile	Ser	Thr	Trp	His	Thr
	65					70					75					80
65	Val	Met	Phe	Trp	Gly	Gly	Lys	Ala	Phe	Phe	Val	Trp	Tyr	Arg	Leu	Ile
					85					90					95	
70	Val	Pro	Leu	Gln	Tyr	Leu	Pro	Leu	Gly	Lys	Val	Leu	Leu	Leu	Phe	Thr
				100					105					110		
75	Val	Ala	Asp	Met	Val	Ser	Ser	Tyr	Trp	Leu	Ala	Leu	Thr	Phe	Gln	Ala
			115					120					125			

Asn Tyr Val Val Glu Glu Val Gln Trp Pro Leu Pro Asp Glu Asn Gly
 130 135 140
 5 Ile Ile Gln Lys Asp Trp Ala Ala Met Gln Val Glu Thr Thr Gln Asp
 145 150 155 160
 Tyr Ala His Asp Ser His Leu Trp Thr Ser Ile Thr Gly Ser Leu Asn
 165 170 175
 10 Tyr Gln Xaa Val His His Leu Phe Pro His
 180 185

(2) INFORMATION FOR SEQ ID NO:15:

15 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 5 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: peptide

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

His Xaa Xaa His His
 1 5

30 (2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 446 amino acids
 35 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: peptide

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Met Ala Ala Gln Ile Lys Lys Tyr Ile Thr Ser Asp Glu Leu Lys Asn
 1 5 10 15
 50 His Asp Lys Pro Gly Asp Leu Trp Ile Ser Ile Gln Gly Lys Ala Tyr
 20 25 30
 Asp Val Ser Asp Trp Val Lys Asp His Pro Gly Gly Ser Phe Pro Leu
 35 40 45
 55 Lys Ser Leu Ala Gly Gln Glu Val Thr Asp Ala Phe Val Ala Phe His
 50 55 60
 Pro Ala Ser Thr Trp Lys Asn Leu Asp Lys Phe Phe Thr Gly Tyr Tyr
 65 70 75 80
 60 Leu Lys Asp Tyr Ser Val Ser Glu Val Ser Lys Val Tyr Arg Lys Leu
 85 90 95

	Val	Phe	Glu	Phe	Ser	Lys	Met	Gly	Leu	Tyr	Asp	Lys	Lys	Gly	His	Ile	
				100					105					110			
5	Met	Phe	Ala	Thr	Leu	Cys	Phe	Ile	Ala	Met	Leu	Phe	Ala	Met	Ser	Val	
			115					120					125				
	Tyr	Gly	Val	Leu	Phe	Cys	Glu	Gly	Val	Leu	Val	His	Leu	Phe	Ser	Gly	
10		130					135					140					
	Cys	Leu	Met	Gly	Phe	Leu	Trp	Ile	Gln	Ser	Gly	Trp	Ile	Gly	His	Asp	
	145					150					155					160	
15	Ala	Gly	His	Tyr	Met	Val	Val	Ser	Asp	Ser	Arg	Leu	Asn	Lys	Phe	Met	
					165					170					175		
	Gly	Ile	Phe	Ala	Ala	Asn	Cys	Leu	Ser	Gly	Ile	Ser	Ile	Gly	Trp	Trp	
				180					185					190			
20	Lys	Trp	Asn	His	Asn	Ala	His	His	Ile	Ala	Cys	Asn	Ser	Leu	Glu	Tyr	
			195					200					205				
	Asp	Pro	Asp	Leu	Gln	Tyr	Ile	Pro	Phe	Leu	Val	Val	Ser	Ser	Lys	Phe	
25		210					215					220					
	Phe	Gly	Ser	Leu	Thr	Ser	His	Phe	Tyr	Glu	Lys	Arg	Leu	Thr	Phe	Asp	
	225					230					235					240	
30	Ser	Leu	Ser	Arg	Phe	Phe	Val	Ser	Tyr	Gln	His	Trp	Thr	Phe	Tyr	Pro	
					245					250					255		
	Ile	Met	Cys	Ala	Ala	Arg	Leu	Asn	Met	Tyr	Val	Gln	Ser	Leu	Ile	Met	
				260					265					270			
35	Leu	Leu	Thr	Lys	Arg	Asn	Val	Ser	Tyr	Arg	Ala	Gln	Glu	Leu	Leu	Gly	
			275					280					285				
	Cys	Leu	Val	Phe	Ser	Ile	Trp	Tyr	Pro	Leu	Leu	Val	Ser	Cys	Leu	Pro	
40		290					295					300					
	Asn	Trp	Gly	Glu	Arg	Ile	Met	Phe	Val	Ile	Ala	Ser	Leu	Ser	Val	Thr	
	305					310					315					320	
45	Gly	Met	Gln	Gln	Val	Gln	Phe	Ser	Leu	Asn	His	Phe	Ser	Ser	Ser	Val	
					325					330					335		
	Tyr	Val	Gly	Lys	Pro	Lys	Gly	Asn	Asn	Trp	Phe	Glu	Lys	Gln	Thr	Asp	
				340				345						350			
50	Gly	Thr	Leu	Asp	Ile	Ser	Cys	Pro	Pro	Trp	Met	Asp	Trp	Phe	His	Gly	
			355					360					365				
	Gly	Leu	Gln	Phe	Gln	Ile	Glu	His	His	Leu	Phe	Pro	Lys	Met	Pro	Arg	
55		370					375					380					
	Cys	Asn	Leu	Arg	Lys	Ile	Ser	Pro	Tyr	Val	Ile	Glu	Leu	Cys	Lys	Lys	
	385					390					395					400	
60	His	Asn	Leu	Pro	Tyr	Asn	Tyr	Ala	Ser	Phe	Ser	Lys	Ala	Asn	Glu	Met	
					405					410					415		

Thr Leu Arg Thr Leu Arg Asn Thr Ala Leu Gln Ala Arg Asp Ile Thr
420 425 430

5 Lys Pro Leu Pro Lys Asn Leu Val Trp Glu Ala Leu His Thr
435 440 445

(2) INFORMATION FOR SEQ ID NO:17:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 359 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: peptide

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Met Leu Thr Ala Glu Arg Ile Lys Phe Thr Gln Lys Arg Gly Phe Arg
1 5 10 15
25 Arg Val Leu Asn Gln Arg Val Asp Ala Tyr Phe Ala Glu His Gly Leu
20 25 30
Thr Gln Arg Asp Asn Pro Ser Met Tyr Leu Lys Thr Leu Ile Ile Val
35 40 45
30 Leu Trp Leu Phe Ser Ala Trp Ala Phe Val Leu Phe Ala Pro Val Ile
50 55 60
35 Phe Pro Val Arg Leu Leu Gly Cys Met Val Leu Ala Ile Ala Leu Ala
65 70 75 80
Ala Phe Ser Phe Asn Val Gly His Asp Ala Asn His Asn Ala Tyr Ser
85 90 95
40 Ser Asn Pro His Ile Asn Arg Val Leu Gly Met Thr Tyr Asp Phe Val
100 105 110
Gly Leu Ser Ser Phe Leu Trp Arg Tyr Arg His Asn Tyr Leu His His
115 120 125
45 Thr Tyr Thr Asn Ile Leu Gly His Asp Val Glu Ile His Gly Asp Gly
130 135 140
50 Ala Val Arg Met Ser Pro Glu Gln Glu His Val Gly Ile Tyr Arg Phe
145 150 155 160
Gln Gln Phe Tyr Ile Trp Gly Leu Tyr Leu Phe Ile Pro Phe Tyr Trp
165 170 175
55 Phe Leu Tyr Asp Val Tyr Leu Val Leu Asn Lys Gly Lys Tyr His Asp
180 185 190
His Lys Ile Pro Pro Phe Gln Pro Leu Glu Leu Ala Ser Leu Leu Gly
195 200 205
60 Ile Lys Leu Leu Trp Leu Gly Tyr Val Phe Gly Leu Pro Leu Ala Leu
210 215 220

Gly Phe Ser Ile Pro Glu Val Leu Ile Gly Ala Ser Val Thr Tyr Met
 225 230 235 240
 5 Thr Tyr Gly Ile Val Val Cys Thr Ile Phe Met Leu Ala His Val Leu
 245 250 255
 Glu Ser Thr Glu Phe Leu Thr Pro Asp Gly Glu Ser Gly Ala Ile Asp
 260 265 270
 10 Asp Glu Trp Ala Ile Cys Gln Ile Arg Thr Thr Ala Asn Phe Ala Thr
 275 280 285
 15 Asn Asn Pro Phe Trp Asn Trp Phe Cys Gly Gly Leu Asn His Gln Val
 290 295 300
 Thr His His Leu Phe Pro Asn Ile Cys His Ile His Tyr Pro Gln Leu
 305 310 315 320
 20 Glu Asn Ile Ile Lys Asp Val Cys Gln Glu Phe Gly Val Glu Tyr Lys
 325 330 335
 Val Tyr Pro Thr Phe Lys Ala Ala Ile Ala Ser Asn Tyr Arg Trp Leu
 340 345 350
 25 Glu Ala Met Gly Lys Ala Ser
 355

30 (2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 365 amino acids
 (B) TYPE: amino acid
 35 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: peptide

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

45 Met Thr Ser Thr Thr Ser Lys Val Thr Phe Gly Lys Ser Ile Gly Phe
 1 5 10 15
 Arg Lys Glu Leu Asn Arg Arg Val Asn Ala Tyr Leu Glu Ala Glu Asn
 20 25 30
 50 Ile Ser Pro Arg Asp Asn Pro Pro Met Tyr Leu Lys Thr Ala Ile Ile
 35 40 45
 Leu Ala Trp Val Val Ser Ala Trp Thr Phe Val Val Phe Gly Pro Asp
 50 55 60
 55 Val Leu Trp Met Lys Leu Leu Gly Cys Ile Val Leu Gly Phe Gly Val
 65 70 75 80
 60 Ser Ala Val Gly Phe Asn Ile Ser His Asp Gly Asn His Gly Gly Tyr
 85 90 95

	Ser	Lys	Tyr	Gln	Trp	Val	Asn	Tyr	Leu	Ser	Gly	Leu	Thr	His	Asp	Ala	
				100					105					110			
5	Ile	Gly	Val	Ser	Ser	Tyr	Leu	Trp	Lys	Phe	Arg	His	Asn	Val	Leu	His	
			115					120					125				
	His	Thr	Tyr	Thr	Asn	Ile	Leu	Gly	His	Asp	Val	Glu	Ile	His	Gly	Asp	
		130					135					140					
10	Glu	Leu	Val	Arg	Met	Ser	Pro	Ser	Met	Glu	Tyr	Arg	Trp	Tyr	His	Arg	
	145					150					155					160	
	Tyr	Gln	His	Trp	Phe	Ile	Trp	Phe	Val	Tyr	Pro	Phe	Ile	Pro	Tyr	Tyr	
15				165						170					175		
	Trp	Ser	Ile	Ala	Asp	Val	Gln	Thr	Met	Leu	Phe	Lys	Arg	Gln	Tyr	His	
				180					185					190			
20	Asp	His	Glu	Ile	Pro	Ser	Pro	Thr	Trp	Val	Asp	Ile	Ala	Thr	Leu	Leu	
			195					200					205				
	Ala	Phe	Lys	Ala	Phe	Gly	Val	Ala	Val	Phe	Leu	Ile	Ile	Pro	Ile	Ala	
		210					215					220					
25	Val	Gly	Tyr	Ser	Pro	Leu	Glu	Ala	Val	Ile	Gly	Ala	Ser	Ile	Val	Tyr	
	225					230					235					240	
	Met	Thr	His	Gly	Leu	Val	Ala	Cys	Val	Val	Phe	Met	Leu	Ala	His	Val	
30					245					250					255		
	Ile	Glu	Pro	Ala	Glu	Phe	Leu	Asp	Pro	Asp	Asn	Leu	His	Ile	Asp	Asp	
				260					265					270			
35	Glu	Trp	Ala	Ile	Ala	Gln	Val	Lys	Thr	Thr	Val	Asp	Phe	Ala	Pro	Asn	
			275					280					285				
	Asn	Thr	Ile	Ile	Asn	Trp	Tyr	Val	Gly	Gly	Leu	Asn	Tyr	Gln	Thr	Val	
		290					295					300					
40	His	His	Leu	Phe	Pro	His	Ile	Cys	His	Ile	His	Tyr	Pro	Lys	Ile	Ala	
		305				310					315					320	
	Pro	Ile	Leu	Ala	Glu	Val	Cys	Glu	Glu	Phe	Gly	Val	Asn	Tyr	Ala	Val	
45					325					330					335		
	His	Gln	Thr	Phe	Phe	Gly	Ala	Leu	Ala	Ala	Asn	Tyr	Ser	Trp	Leu	Lys	
				340					345					350			
50	Lys	Met	Ser	Ile	Asn	Pro	Glu	Thr	Lys	Ala	Ile	Glu	Gln				
			355					360					365				

(2) INFORMATION FOR SEQ ID NO:19:

- 55 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 35 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- 60 (ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

5 CCAAGCTTCT GCAGGAGCTC TTTTTTTTTT TTTT 35

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Synthetic oligonucleotide"

(ix) FEATURE:

- 20 (A) NAME/KEY: misc_feature
(B) LOCATION: 21
(D) OTHER INFORMATION: /number= 1
/note= "N=Inosine or Cytosine"

(ix) FEATURE:

- 25 (A) NAME/KEY: misc_feature
(B) LOCATION: 27
(D) OTHER INFORMATION: /number= 2
30 /note= "N=Inosine or Cytosine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

35 CUACUACUAC UACAYCAYAC NTAYACNAAY AT 32

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- 40 (A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Synthetic oligonucleotide"

(ix) FEATURE:

- 50 (A) NAME/KEY: misc_feature
(B) LOCATION: 13
(D) OTHER INFORMATION: /number= 1
/note= "N=Inosine or Cytosine"

(ix) FEATURE:

- 55 (A) NAME/KEY: misc_feature
(B) LOCATION: 19
(D) OTHER INFORMATION: /number= 2
60 /note= "N=Inosine or Cytosine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CAUCAUCAUC AUNGGRAANA RRTGRTG

27

(2) INFORMATION FOR SEQ ID NO:22:

- 5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
10 (D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CUACUACUAC UAGGAGTCCT CTACGGTGTT TTG

33

20

(2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
25 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

35 CAUCAUCAUC AUATGATGCT CAAGCTGAAA CTG

33

(2) INFORMATION FOR SEQ ID NO:24:

- 40 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

45

(ii) MOLECULE TYPE: peptide

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Gln Xaa Xaa His His
1 5

55

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
60 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CUACUACUAC UACTCGAGCA AGATGGGAAC GGACCAAGG

39

10

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39 base pairs

(B) TYPE: nucleic acid

15

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

25

CAUCAUCAUC AUCTCGAGCT ACTCTTCCTT GGGACGGAG

39

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 47 base pairs

(B) TYPE: nucleic acid

30

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: other nucleic acid

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CUACUACUAC UATCTAGACT CGAGACCATG GCTGCTGCTC CAGTGTG

47

(2) INFORMATION FOR SEQ ID NO:28:

45

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40 base pairs

(B) TYPE: nucleic acid

50

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CAUCAUCAUC AUAGGCCTCG AGTTACTGCG CCTTACCCAT

60

40

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CUACUACUA CUAGGATCCA TGGCACCTCC CAACACT

37

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CAUCAUCAU CAUGGTACCT CGAGTTACTT CTTGAAAAAG AC

42

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1219 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid (Edited Contig 2692004)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GCACGCCGAC CGGCGCCGGG AGATCCTGGC AAAGTATCCA GAGATAAAGT CCTTGATGAA 60
ACCTGATCCC AATTGATAT GGATTATAAT TATGATGGTT CTCACCCAGT TGGGTGCATT 120
TTACATAGTA AAAGACTTGG ACTGGAATG GGTCAATTTT GGGGCCTATG CGTTTGGCAG 180
TTGCATTAA CACTCAATGA CTCTGGCTAT TCATGAGATT GCCCACAATG CTGCCTTTGG 240
CAACTGCAAA GCAATGTGGA ATCGCTGGTT TGGAATGTTT GCTAATCTTC CTATTGGGAT 300
TCCATATTCA ATTTCTTTA AGAGGTATCA CATGGATCAT CATCGGTACC TTGGAGCTGA 360
TGGCGTCGAT GTAGATATTC CTACCGATTT TGAGGGCTGG TTCTTCTGTA CCGCTTTCAG 420
AAAGTTTATA TGGGTATTTC TTCAGCCTCT CTTTTATGCC TTTGACCTC TGTTTCATCAA 480

5 CCCCCAACCA ATTACGTATC TGGAAGTTAT CAATACCGTG GCACAGGTCA CTTTGTACAT 540
 TTTAATTTAT TACTTTTTGG GAATTAAATC CTTAGTCTAC ATGTTGGCAG CATCTTTACT 600
 TGGCCTGGGT TTGCACCCAA TTTCTGGACA TTTTATAGCT GAGCATTACA TGTCTTAAA 660
 GGGTCATGAA ACTTACTCAT ATTATGGGCC TCTGAATTTA CTTACCTTCA ATGTGGGTTA 720
 10 TCATAATGAA CATCATGATT TCCCCAACAT TCCTGGAAAA AGTCTTCCAC TGGTGAGGAA 780
 AATAGCAGCT GAATACTATG ACAACCTCCC TCACTACAAT TCCTGGATAA AAGTACTGTA 840
 TGATTTTGTG ATGGATGATA CAATAAGTCC CTA CTCAAGA ATGAAGAGGC ACCAAAAAGG 900
 15 AGAGATGGTG CTGGAGTAAA TATCATTAGT GCCAAAGGGA TTCTTCTCCA AAACCTTAGA 960
 TGATAAAATG GAATTTTTCG ATTATTAAAC TTGAGACCAG TGATGCTCAG AAGCTCCCCT 1020
 20 GGCACAATTT CAGAGTAAGA GCTCGGTGAT ACCAAGAAGT GAATCTGGCT TTTAAACAGT 1080
 CAGCCTGACT CTGTACTGCT CAGTTTCACT CACAGGAAAC TTGTGACTTG TGTATTATCG 1140
 TCATTGAGGA TGTTTCACTC ATGTCTGTCA TTTTATAAGC ATATCATTTA AAAAGCTTCT 1200
 25 AAAAAGCTAT TTCGCCAGG 1219

(2) INFORMATION FOR SEQ ID NO:32:

30

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 655 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

35

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid (Edited Contig 2153526)

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

45 TTACCTTCTA CGTCCGCTTC TTCCTCACTT ATGTGCCACT ATTGGGGCTG AAAGCTTCCT 60
 GGGCCTTTTC TTCATAGTCA GGTTCTGGA AAGCAACTGG TTTGTGTGGG TGACACAGAT 120
 GAACCATATT CCCATGCACA TTGATCATGA CCGGAACATG GACTGGGTTT CCACCCAGCT 180
 50 CCAGGCCACA TGCAATGTCC ACAAGTCTGC CTTCAATGAC TGGTTCAGTG GACACCTCAA 240
 CTTCCAGATT GAGCACCATC TTTTCCAC GATGCCTCGA CACAATTACC ACAAAGTGGC 300
 55 TCCCCTGGTG CAGTCCTTGT GTGCCAAGCA TGGCATAGAG TACCAGTCCA AGCCCCTGCT 360
 GTCAGCCTTC GCCGACATCA TCCACTCACT AAAGGAGTCA GGGCAGCTCT GGCTAGATGC 420
 CTATCTTCAC CAATAACAAC AGCCACCCTG CCCAGTCTGG AAGAAGAGGA GGAAGACTCT 480
 60 GGAGCCAAGG CAGAGGGGAG CTTGAGGGAC AATGCCACTA TAGTTTAATA CTCAGAGGGG 540
 GTTGGGTTTG GGGACATAAA GCCTCTGACT CAACTCCTC CCTTTTATCT TCTAGCCACA 600

GTTCTAAGAC CCAAAGTGGG GGGTGGACAC AGAAGTCCCT AGGAGGGAAG GAGCT 655

5 (2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 304 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid (Edited Contig 3506132)

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GTCTTTTACT TTGGCAATGG CTGGATTCTT ACCCTCATCA CGGCCTTTGT CCTTGCTACC 60
TCTCAGGCCC AAGCTGGATG GCTGCAACAT GATTATGGCC ACCTGTCTGT CTACAGAAAA 120
20 CCCAAGTGGG ACCACCTTGT CCACAAATTC GTCATTGGCC ACTTAAAGGG TGCCTCTGCC 180
AACTGGTGGG ATCATCGCCA CTTCCAGCAC CACGCCAAGC CTAACATCTT CCACAAGGAT 240
25 CCCGATGTGA ACATGCTGCA CGTGTTTGTT CTGGGCGAAT GGCAGCCCAT CGAGTACGGC 300
AAGA 304

30 (2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

- 35 (A) LENGTH: 918 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid (Edited Contig 3854933)

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CAGGGACCTA CCCC GCGCTA CTTACCTGG GACGAGGTGG CCCAGCGCTC AGGGTGCGAG 60
GAGCGGTGGC TAGTGATCGA CCGTAAGGTG TACAACATCA GCGAGTTCAC CCGCCGGCAT 120
45 CCAGGGGGCT CCCGGGTCAT CAGCCACTAC GCCGGGCAGG ATGCCACGGA TCCCTTTGTG 180
GCCTTCCACA TCAACAAGGG CTTGTGAAG AAGTATATGA ACTCTCTCCT GATTGGAGAA 240
50 CTGTCTCCAG AGCAGCCCAG CTTTGAGCCC ACCAAGAATA AAGAGCTGAC AGATGAGTTC 300
CGGGAGCTGC GGGCCACAGT GGAGCGGATG GGGCTCATGA AGGCCAACCA TGTCTTCTTC 360
CTGCTGTACC TGCTGCACAT CTTGCTGCTG GATGGTGCAG CCTGGCTCAC CTTTGGGTTC 420
55 TTTGGGACGT CCTTTTGGC CTTCTCCTC TGTGCGGTGC TGCTCAGTGC AGTTCAGGCC 480
CAGGCTGGCT GGCTGCAGCA TGACTTTGGG CACCTGTCGG TCTTCAGCAC CTCAAAGTGG 540
60 AACCATCTGC TACATCATTT TGTGATTGGC CACCTGAAGG GGGCCCCCGC CAGTTGGTGG 600
AACCACATGC ACTTCCAGCA CCATGCCAAG CCCAACTGCT TCCGCAAAGA CCCAGACATC 660

AACATGCATC CCTTCTTCTT TGCCTTGGGG AAGATCCTCT CTGTGGAGCT TGGGAAACAG 720
 5 AAGAAAAAAT ATATGCCGTA CAACCACCAG CACARATACT TCTTCCTAAT TGGGCCCCCA 780
 GCCTTGCTGC CTCTCTACTT CCAGTGGTAT ATTTTCTATT TTGTTATCCA GCGAAAGAAG 840
 TGGGTGGACT TGGCCTGGAT CAGCAAACAG GAATACGATG AAGCCGGGCT TCCATTGTCC 900
 10 ACCGCAAATG CTTCTAAA 918

(2) INFORMATION FOR SEQ ID NO:35:

15 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1686 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 20 (ii) MOLECULE TYPE: other nucleic acid (Edited Contig 2511785)
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:
 25 GCCACTTAAA GGGTGCCTCT GCCAACTGGT GGAATCATCG CCACTTCCAG CACCACGCCA 60
 AGCCTAACAT CTTCCACAAG GATCCCGATG TGAACATGCT GCACGTGTTT GTTCTGGGCG 120
 30 AATGGCAGCC CATCGAGTAC GGCAAGAAGA AGCTGAAATA CCTGCCCTAC AATCACCAGC 180
 ACGAATACTT CTTCTGATT GGGCCGCCGC TGCTCATCCC CATGTATTTC CAGTACCAGA 240
 35 TCATCATGAC CATGATCGTC CATAAGAACT GGGTGGACCT GGCTGGGCC GTCAGCTACT 300
 ACATCCGGTT CTTTCATCACC TACATCCCTT TCTACGGCAT CCTGGGAGCC CTCCTTTTCC 360
 TCAACTTCAT CAGGTTCTTG GAGAGCCACT GGTTTGTGTG GGTCACACAG ATGAATCACA 420
 40 TCGTCATGGA GATTGACCAG GAGGCCTACC GTGACTGGTT CAGTAGCCAG CTGACAGCCA 480
 CCTGCAACGT GGAGCAGTCC TTCTTCAACG ACTGGTTCAG TGGACACCTT AACTTCCAGA 540
 45 TTGAGCACCA CCTCTTCCCC ACCATGCCCC GGCACAACTT ACACAAGATC GCCCCGCTGG 600
 TGAAGTCTCT ATGTGCCAAG CATGGCATTG AATACCAGGA GAAGCCGCTA CTGAGGGCCC 660
 TGCTGGACAT CATCAGGTCC CTGAAGAAGT CTGGGAAGCT GTGGCTGGAC GCCTACCTTC 720
 50 ACAAATGAAG CCACAGCCCC CGGGACACCG TGGGAAGGG GTGCAGGTGG GGTGATGGCC 780
 AGAGGAATGA TGGGCTTTTG TTCTGAGGGG TGTCCGAGAG GCTGGTGTAT GCACTGCTCA 840
 CGGACCCCAT GTTGATCTT TCTCCCTTC TCCTCTCCTT TTTCTCTTCA CATCTCCCCC 900
 55 ATAGCACCCT GCCCTCATGG GACCTGCCCT CCCTCAGCCG TCAGCCATCA GCCATGGCCC 960
 TCCCAGTGCC TCCTAGCCCC TTCTTCCAAG GAGCAGAGAG GTGGCCACCG GGGGTGGCTC 1020
 60 TGTCTACCT CCACTCTCTG CCCCTAAAGA TGGGAGGAGA CCAGCGGTCC ATGGGTCTGG 1080
 CCTGTGAGTC TCCCCTTGCA GCCTGGTCAC TAGGCATCAC CCCCCTTTG GTTCTTCAGA 1140

5 TGCTCTTGGG GTTCATAGGG GCAGGTCCTA GTCGGGCAGG GCCCCTGACC CTCCCGGCCT 1200
 GGCTTCACTC TCCCTGACGG CTGCCATTGG TCCACCCTTT CATAGAGAGG CCTGCTTTGT 1260
 TACAAAGCTC GGGTCTCCCT CCTGCAGCTC GGTTAAGTAC CCGAGGCCTC TCTTAAGATG 1320
 TCCAGGGCCC CAGGCCCCGCG GGCACAGCCA GCCCAAACCT TGGGCCCTGG AAGAGTCCTC 1380
 10 CACCCCATCA CTAGAGTGCT CTGACCCTGG GCTTTCACGG GCCCATTCC ACCGCCTCCC 1440
 CAACTTGAGC CTGTGACCTT GGGACCAAAG GGGGAGTCCC TCGTCTCTTG TGA CTAGCA 1500
 GAGGCAGTGG CCACGTTT CAG GGAGGGGCGG GCTGGCCTGG AGGCTCAGCC CACCCTCCAG 1560
 15 CTTTTCTCA GGGTGTCTG AGGTCCAAGA TTCTGGAGCA ATCTGACCCT TCTCCAAAGG 1620
 CTCTGTTATC AGCTGGGCAG TGCCAGCCAA TCCCTGGCCA TTTGGCCCCA GGGGACGTGG 1680
 20 GCCCTG 1686

(2) INFORMATION FOR SEQ ID NO:36:

25 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1843 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 30 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: other nucleic acid (Contig 2535)
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

35 GTCTTTTACT TTGGCAATGG CTGGATTCCT ACCCTCATCA CGGCCTTTGT CCTTGCTACC 60
 TCTCAGGCCC AAGCTGGATG GCTGCAACAT GATTATGGCC ACCTGTCTGT CTACAGAAAA 120
 40 CCCAAGTGGG ACCACCTTGT CCACAAATTC GTCATTGGCC ACTTAAAGGG TGCTCTGCC 180
 AACTGGTGGG ATCATCGCCA CTTCCAGCAC CAGCCAAGC CTAACATCTT CCACAAGGAT 240
 45 CCCGATGTGA ACATGCTGCA CGTGTTTGTT CTGGGCGAAT GGCAGCCCAT CGAGTACGGC 300
 AAGAAGAAGC TGAAATACCT GCCCTACAAT CACCAGCACG AATACTTCTT CCTGATTGGG 360
 CCGCCGCTGC TCATCCCCAT GTATTTCCAG TACCAGATCA TCATGACCAT GATCGTCCAT 420
 50 AAGAACTGGG TGGACCTGGC CTGGGCCGTC AGCTACTACA TCCGGTTCTT CATCACCTAC 480
 ATCCCTTTCT ACGGCATCCT GGGAGCCCTC CTTTTCTCA ACTTCATCAG GTTCCTGGAG 540
 AGCCACTGGT TTGTGTGGGT CACACAGATG AATCACATCG TCATGGAGAT TGACCAGGAG 600
 55 GCCTACCGTG ACTGGTTCAG TAGCCAGCTG ACAGCCACCT GCAACGTGGA GCAGTCCTTC 660
 TTCAACGACT GGTTCAGTGG ACACCTTAAC TTCCAGATTG AGCACCACCT CTTCCCCACC 720
 60 ATGCCCCGGC ACAACTTACA CAAGATCGCC CCGCTGGTGA AGTCTCTATG TGCCAAGCAT 780
 GGCATTGAAT ACCAGGAGAA GCCGCTACTG AGGGCCCTGC TGGACATCAT CAGGTCCCTG 840

5 AAGAAGTCTG GGAAGCTGTG GCTGGACGCC TACCTTCACA AATGAAGCCA CAGCCCCCGG 900
 GACACCGTGG GGAAGGGGTG CAGGTGGGGT GATGGCCAGA GGAATGATGG GCTTTTGTTC 960
 TGAGGGGTGT CCGAGAGGCT GGTGTATGCA CTGCTCACGG ACCCCATGTT GGATCTTTCT 1020
 CCCTTTCTCC TCTCCTTTT CTCTTCACAT CTCCCCATA GCACCCTGCC CTCATGGGAC 1080
 10 CTGCCCTCCC TCAGCCGTCA GCCATCAGCC ATGGCCCTCC CAGTGCCTCC TAGCCCTTC 1140
 TTCCAAGGAG CAGAGAGGTG GCCACCGGG GTGGCTCTGT CCTACCTCCA CTCTCTGCCC 1200
 CTAAAGATGG GAGGAGACCA GCGGTCCATG GGTCTGGCCT GTGAGTCTCC CTTGCAGCC 1260
 15 TGGTCACTAG GCATCACCCC CGCTTTGGTT CTTCAGATGC TCTTGGGGTT CATAGGGGCA 1320
 GGTCTAGTC GGGCAGGGCC CCTGACCCTC CCGGCCTGGC TTTACTCTCC CTGACGGCTG 1380
 20 CCATTGGTCC ACCCTTTCAT AGAGAGGCCT GCTTTGTTAC AAAGCTCGGG TCTCCCTCCT 1440
 GCAGCTCGGT TAAGTACCCG AGGCCTCTCT TAAGATGTCC AGGGCCCCAG GCCCGCGGGC 1500
 ACAGCCAGCC CAAACCTTGG GCCCTGGAAG AGTCCTCCAC CCCATCACTA GAGTGCTCTG 1560
 25 ACCCTGGGCT TTCACGGGCC CCATTCCACC GCCTCCCCAA CTTGAGCCTG TGACCTTGGG 1620
 ACCAAAGGGG GAGTCCCTCG TCTCTTGTA CTCAGCAGAG GCAGTGGCCA CGTTCAGGGA 1680
 30 GGGGCCGGCT GGCCTGGAGG CTCAGCCCAC CCTCCAGCTT TTCCTCAGGG TGTCTGAGG 1740
 TCCAAGATTC TGGAGCAATC TGACCCTTCT CCAAAGGCTC TGTATCAGC TGGGCAGTGC 1800
 35 CAGCCAATCC CTGGCCATTT GGCCCCAGGG GACGTGGGCC CTG 1843

(2) INFORMATION FOR SEQ ID NO:37:

40 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 2257 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 45 (ii) MOLECULE TYPE: other nucleic acid (Edited Contig 253538a)
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:
 50 CAGGGACCTA CCCC GCGCTA CTTACCTGG GACGAGGTGG CCCAGCGCTC AGGGTGCAG 60
 GAGCGGTGGC TAGTGATCGA CCGTAAGGTG TACAACATCA GCGAGTTCAC CCGCCGGCAT 120
 CCAGGGGGCT CCCGGGTCAT CAGCCACTAC GCCGGGCAGG ATGCCACGGA TCCCTTTGTG 180
 55 GCCTTCCACA TCAACAAGGG CCTTGTGAAG AAGTATATGA ACTCTCTCCT GATTGGAGAA 240
 CTGTCTCCAG AGCAGCCCAG CTTTGTAGCCC ACCAAGAATA AAGAGCTGAC AGATGAGTTC 300
 CGGGAGCTGC GGGCCACAGT GGAGCGGATG GGGCTCATGA AGGCCAACCA TGTCTTCTTC 360
 60 CTGCTGTACC TGCTGCACAT CTTGCTGCTG GATGGTGCAG CTTGGCTCAC CCTTTGGGTC 420

	TTTGGGACGT CCTTTTTGCC CTCCTCCTC TGTGCGGTGC TGCTCAGTGC AGTTCAGCAG	480
	GCCCAAGCTG GATGGCTGCA ACATGATTAT GGCCACCTGT CTGTCTACAG AAAACCCAAG	540
5	TGGAACCACC TTGTCCACAA ATTCGTCATT GGCCACTTAA AGGGTGCCTC TGCCAACTGG	600
	TGGAATCATC GCCACTTCCA GCACCACGCC AAGCCTAACA TCTTCCACAA GGATCCCGAT	660
10	GTGAACATGC TGCACGTGTT TGTTCCTGGGC GAATGGCAGC CCATCGAGTA CGGCAAGAAG	720
	AAGCTGAAAT ACCTGCCCTA CAATCACCAG CACGAATACT TCTTCCTGAT TGGGCCGCCG	780
	CTGCTCATCC CCATGTATTT CCAGTACCAG ATCATCATGA CCATGATCGT CCATAAGAAC	840
15	TGGGTGGACC TGGCCTGGGC CGTCAGCTAC TACATCCGGT TCTTCATCAC CTACATCCCT	900
	TTCTACGGCA TCCTGGGAGC CCTCCTTTTC CTCAACTTCA TCAGGTTTCCT GGAGAGCCAC	960
20	TGGTTTGTGT GGGTCACACA GATGAATCAC ATCGTCATGG AGATTGACCA GGAGGCCTAC	1020
	CGTGACTGGT TCAGTAGCCA GCTGACAGCC ACCTGCAACG TGGAGCAGTC CTTCTTCAAC	1080
	GACTGGTTCA GTGGACACCT TAACTTCCAG ATTGAGCACC ACCTCTTCCC CACCATGCCC	1140
25	CGGCACAAC TACACAAGAT CGCCCCGCTG GTGAAGTCTC TATGTGCCAA GCATGGCATT	1200
	GAATACCAGG AGAAGCCGCT ACTGAGGGCC CTGCTGGACA TCATCAGGTC CCTGAAGAAG	1260
30	TCTGGGAAGC TGTGGCTGGA CGCCTACCTT CACAAATGAA GCCACAGCCC CCGGGACACC	1320
	GTGGGGAAGG GGTGCAGGTG GGGTGATGGC CAGAGGAATG ATGGGCTTTT GTTCTGAGGG	1380
	GTGTCCGAGA GGCTGGTGTA TGCACGTCTC ACGGACCCCA TGTGGATCT TTCTCCCTTT	1440
35	CTCCTCTCCT TTTTCTCTTC ACATCTCCCC CATAGCACCC TGCCCTCATG GGACCTGCCC	1500
	TCCCTCAGCC GTCAGCCATC AGCCATGGCC CTCCCAGTGC CTCCTAGCCC CTTCTTCCAA	1560
40	GGAGCAGAGA GGTGGCCACC GGGGGTGGCT CTGTCCTACC TCCACTCTCT GCCCCTAAAG	1620
	ATGGGAGGAG ACCAGCGGTC CATGGGTCTG GCCTGTGAGT CTCCCCTTGC AGCCTGGTCA	1680
	CTAGGCATCA CCCCCGCTTT GTTCTTTCAG ATGCTCTTGG GGTTCATAGG GGCAGGTCCT	1740
45	AGTCGGGCAG GGCCCCTGAC CCTCCCGGCC TGGCTTCACT CTCCCTGACG GCTGCCATTG	1800
	GTCCACCCTT TCATAGAGAG GCCTGCTTTG TTACAAAGCT CGGGTCTCCC TCCTGCAGCT	1860
50	CGGTTAAGTA CCCGAGGCCT CTCTTAAGAT GTCCAGGGCC CCAGGCCCGC GGGCACAGCC	1920
	AGCCCAAACC TTGGGCCCTG GAAGAGTCCT CCACCCCATC ACTAGAGTGC TCTGACCCTG	1980
	GGCTTTCACG GGCCCCATTC CACCGCCTCC CCAACTTGAG CCTGTGACCT TGGGACCAAA	2040
55	GGGGGAGTCC CTCGTCTCTT GTGACTCAGC AGAGGCAGTG GCCACGTTCA GGGAGGGGCC	2100
	GGCTGGCCTG GAGGCTCAGC CCACCCTCCA GCTTTTCCTC AGGGTGTCTT GAGGTCCAAG	2160
60	ATTCTGGAGC AATCTGACCC TTCTCCAAAG GCTCTGTTAT CAGCTGGGCA GTGCCAGCCA	2220
	ATCCCTGGCC ATTTGGCCCC AGGGGACGTG GGCCCTG	2257

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 411 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: amino acid (Translation of Contig 2692004)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

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15 His Ala Asp Arg Arg Arg Glu Ile Leu Ala Lys Tyr Pro Glu Ile
   1           5           10           15
Lys Ser Leu Met Lys Pro Asp Pro Asn Leu Ile Trp Ile Ile Ile
   20           25           30
Met Met Val Leu Thr Gln Leu Gly Ala Phe Tyr Ile Val Lys Asp
   35           40           45
20 Leu Asp Trp Lys Trp Val Ile Phe Gly Ala Tyr Ala Phe Gly Ser
   50           55           60
Cys Ile Asn His Ser Met Thr Leu Ala Ile His Glu Ile Ala His
   65           70           75
25 Asn Ala Ala Phe Gly Asn Cys Lys Ala Met Trp Asn Arg Trp Phe
   80           85           90
Gly Met Phe Ala Asn Leu Pro Ile Gly Ile Pro Tyr Ser Ile Ser
   95          100          105
30 Phe Lys Arg Tyr His Met Asp His His Arg Tyr Leu Gly Ala Asp
   110          115          120
Gly Val Asp Val Asp Ile Pro Thr Asp Phe Glu Gly Trp Phe Phe
   125          130          135
Cys Thr Ala Phe Arg Lys Phe Ile Trp Val Ile Leu Gln Pro Leu
   140          145          150
35 Phe Tyr Ala Phe Arg Pro Leu Phe Ile Asn Pro Lys Pro Ile Thr
   155          160          165
Tyr Leu Glu Val Ile Asn Thr Val Ala Gln Val Thr Phe Asp Ile
   170          175          180
Leu Ile Tyr Tyr Phe Leu Gly Ile Lys Ser Leu Val Tyr Met Leu
   185          190          195
40 Ala Ala Ser Leu Leu Gly Leu Gly Leu His Pro Ile Ser Gly His
   200          205          210
Phe Ile Ala Glu His Tyr Met Phe Leu Lys Gly His Glu Thr Tyr
   215          220          225
45 Ser Tyr Tyr Gly Pro Leu Asn Leu Leu Thr Phe Asn Val Gly Tyr
   230          235          240
His Asn Glu His His Asp Phe Pro Asn Ile Pro Gly Lys Ser Leu
   245          250          255
Pro Leu Val Arg Lys Ile Ala Ala Glu Tyr Tyr Asp Asn Leu Pro
   260          265          270
50 His Tyr Asn Ser Trp Ile Lys Val Leu Tyr Asp Phe Val Met Asp
   275          280          285
Asp Thr Ile Ser Pro Tyr Ser Arg Met Lys Arg His Gln Lys Gly
   290          295          300
55 Glu Met Val Leu Glu *** Ile Ser Leu Val Pro Lys Gly Phe Phe
   305          310          315
Ser Lys Thr Leu Asp Asp Lys Met Glu Phe Leu His Tyr *** Thr
   320          325          330
*** Asp Gln *** Cys Ser Glu Ala Pro Leu Ala Gln Phe Gln Ser
   335          340          345
60 Lys Ser Ser Val Ile Pro Arg Ser Glu Ser Gly Phe *** Thr Val
   350          355          360

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Ser Leu Thr Leu Tyr Cys-Ser Val Ser Leu Thr Gly Asn Leu ***
 365 370 375
 Leu Val Tyr Tyr Arg His *** Gly Cys Phe Thr His Val Cys His
 380 385 390
 5 Phe Ile Ser Ile Ser Phe Lys Lys Leu Leu Lys Ser Tyr Phe Ala
 400 405 410
 Arg

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 218 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid (Translation of Contig 2153526)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

Tyr Leu Leu Arg Pro Leu Leu Pro His Leu Cys Ala Thr Ile Gly
 1 5 10 15
 25 Ala Glu Ser Phe Leu Gly Leu Phe Phe Ile Val Arg Phe Leu Glu
 20 25 30
 Ser Asn Trp Phe Val Trp Val Thr Gln Met Asn His Ile Pro Met
 35 40 45
 His Ile Asp His Asp Arg Asn Met Asp Trp Val Ser Thr Gln Leu
 50 55 60
 30 Gln Ala Thr Cys Asn Val His Lys Ser Ala Phe Asn Asp Trp Phe
 65 70 75
 Ser Gly His Leu Asn Phe Gln Ile Glu His His Leu Phe Pro Thr
 80 85 90
 35 Met Pro Arg His Asn Tyr His Lys Val Ala Pro Leu Val Gln Ser
 95 100 105
 Leu Cys Ala Lys His Gly Ile Glu Tyr Gln Ser Lys Pro Leu Leu
 110 115 120
 Ser Ala Phe Ala Asp Ile Ile His Ser Leu Lys Glu Ser Gly Gln
 125 130 135
 40 Leu Trp Leu Asp Ala Tyr Leu His Gln *** Gln Gln Pro Pro Cys
 140 145 150
 Pro Val Trp Lys Lys Arg Arg Lys Thr Leu Glu Pro Arg Gln Arg
 155 160 165
 45 Gly Ala *** Gly Thr Met Pro Leu *** Phe Asn Thr Gln Arg Gly
 170 175 180
 Leu Gly Leu Gly Thr *** Ser Leu *** Leu Lys Leu Leu Pro Phe
 185 190 195
 Ile Phe *** Pro Gln Phe *** Asp Pro Lys Trp Gly Val Asp Thr
 200 205 210
 50 Glu Val Pro Arg Arg Glu Gly Ala
 215

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 71 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid (Translation of Contig 3506132)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

5

	Val	Phe	Tyr	Phe	Gly	Asn	Gly	Trp	Ile	Pro	Thr	Leu	Ile	Thr	Ala
	1				5					10					15
10	Phe	Val	Leu	Ala	Thr	Ser	Gln	Ala	Gln	Ala	Gly	Trp	Leu	Gln	His
					20					25					30
	Asp	Tyr	Gly	His	Leu	Ser	Val	Tyr	Arg	Lys	Pro	Lys	Trp	Asn	His
					35					40					45
	Leu	Val	His	Lys	Phe	Val	Ile	Gly	His	Leu	Lys	Gly	Ala	Ser	Ala
					50					55					60
15	Asn	Trp	Trp	Asn	His	Arg	His	Phe	Gln	His	His	Ala	Lys	Pro	Asn
					65					70					75
	Leu	Gly	Glu	Trp	Gln	Pro	Ile	Glu	Tyr	Gly	Lys	Xxx			
					80					85					

20

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 306 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: amino acid (Translation of Contig 3854933)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

35	Gln	Gly	Pro	Thr	Pro	Arg	Tyr	Phe	Thr	Trp	Asp	Glu	Val	Ala	Gln
	1				5					10					15
	Arg	Ser	Gly	Cys	Glu	Glu	Arg	Trp	Leu	Val	Ile	Asp	Arg	Lys	Val
					20					25					30
40	Tyr	Asn	Ile	Ser	Glu	Phe	Thr	Arg	Arg	His	Pro	Gly	Gly	Ser	Arg
					35					40					45
	Val	Ile	Ser	His	Tyr	Ala	Gly	Gln	Asp	Ala	Thr	Asp	Pro	Phe	Val
					50					55					60
	Ala	Phe	His	Ile	Asn	Lys	Gly	Leu	Val	Lys	Lys	Tyr	Met	Asn	Ser
					65					70					75
45	Leu	Leu	Ile	Gly	Glu	Leu	Ser	Pro	Glu	Gln	Pro	Ser	Phe	Glu	Pro
					80					85					90
	Thr	Lys	Asn	Lys	Glu	Leu	Thr	Asp	Glu	Phe	Arg	Glu	Leu	Arg	Ala
					95					100					105
50	Thr	Val	Glu	Arg	Met	Gly	Leu	Met	Lys	Ala	Asn	His	Val	Phe	Phe
					110					115					120
	Leu	Leu	Tyr	Leu	Leu	His	Ile	Leu	Leu	Leu	Asp	Gly	Ala	Ala	Trp
					125					130					135
	Leu	Thr	Leu	Trp	Val	Phe	Gly	Thr	Ser	Phe	Leu	Pro	Phe	Leu	Leu
					140					145					150
55	Cys	Ala	Val	Leu	Leu	Ser	Ala	Val	Gln	Ala	Gln	Ala	Gly	Trp	Leu
					155					160					165
	Gln	His	Asp	Phe	Gly	His	Leu	Ser	Val	Phe	Ser	Thr	Ser	Lys	Trp
					170					175					180
	Asn	His	Leu	Leu	His	His	Phe	Val	Ile	Gly	His	Leu	Lys	Gly	Ala
					185					190					195
60	Pro	Ala	Ser	Trp	Trp	Asn	His	Met	His	Phe	Gln	His	His	Ala	Lys
					200					205					210

Pro Asn Cys Phe Arg Lys Asp Pro Asp Ile Asn Met His Pro Phe
 215 220 225
 Phe Phe Ala Leu Gly Lys Ile Leu Ser Val Glu Leu Gly Lys Gln
 230 235 240
 5 Lys Lys Lys Tyr Met Pro Tyr Asn His Gln His Xxx Tyr Phe Phe
 245 250 255
 Leu Ile Gly Pro Pro Ala Leu Leu Pro Leu Tyr Phe Gln Trp Tyr
 260 265 270
 10 Ile Phe Tyr Phe Val Ile Gln Arg Lys Lys Trp Val Asp Leu Ala
 275 280 285
 Trp Ile Ser Lys Gln Glu Tyr Asp Glu Ala Gly Leu Pro Leu Ser
 290 295 300
 Thr Ala Asn Ala Ser Lys
 305
 15

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
 20 (A) LENGTH: 566 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: amino acid (Translation of Contig 2511785)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

30 His Leu Lys Gly Ala Ser Ala Asn Trp Trp Asn His Arg His Phe
 1 5 10 15
 Gln His His Ala Lys Pro Asn Ile Phe His Lys Asp Pro Asp Val
 20 25 30
 35 Asn Met Leu His Val Phe Val Leu Gly Glu Trp Gln Pro Ile Glu
 35 40 45
 Tyr Gly Lys Lys Lys Leu Lys Tyr Leu Pro Tyr Asn His Gln His
 50 55 60
 Glu Tyr Phe Phe Leu Ile Gly Pro Pro Leu Leu Ile Pro Met Tyr
 65 70 75
 40 Phe Gln Tyr Gln Ile Ile Met Thr Met Ile Val His Lys Asn Trp
 80 85 90
 Val Asp Leu Ala Trp Ala Val Ser Tyr Tyr Ile Arg Phe Phe Ile
 95 100 105
 45 Thr Tyr Ile Pro Phe Tyr Gly Ile Leu Gly Ala Leu Leu Phe Leu
 110 115 120
 Asn Phe Ile Arg Phe Leu Glu Ser His Trp Phe Val Trp Val Thr
 125 130 135
 Gln Met Asn His Ile Val Met Glu Ile Asp Gln Glu Ala Tyr Arg
 140 145 150
 50 Asp Trp Phe Ser Ser Gln Leu Thr Ala Thr Cys Asn Val Glu Gln
 155 160 165
 Ser Phe Phe Asn Asp Trp Phe Ser Gly His Leu Asn Phe Gln Ile
 170 175 180
 55 Glu His His Leu Phe Pro Thr Met Pro Arg His Asn Leu His Lys
 185 190 195
 Ile Ala Pro Leu Val Lys Ser Leu Cys Ala Lys His Gly Ile Glu
 200 205 210
 Tyr Gln Glu Lys Pro Leu Leu Arg Ala Leu Leu Asp Ile Ile Arg
 215 220 225
 60 Ser Leu Lys Lys Ser Gly Lys Leu Trp Leu Asp Ala Tyr Leu His
 230 235 240
 Lys *** Ser His Ser Pro Arg Asp Thr Val Gly Lys Gly Cys Arg

				245					250				255
	Trp	Gly	Asp	Gly	Gln	Arg	Asn	Asp	Gly	Leu	Leu	Phe	*** Gly Val
				260						265			270
5	Ser	Glu	Arg	Leu	Val	Tyr	Ala	Leu	Leu	Thr	Asp	Pro	Met Leu Asp
				275						280			285
	Leu	Ser	Pro	Phe	Leu	Ser	Phe	Phe		Ser	Ser	His	Leu Pro His
				290						295			300
	Ser	Thr	Leu	Pro	Ser	Trp	Asp	Leu	Pro	Ser	Leu	Ser	Arg Gln Pro
				305						310			315
10	Ser	Ala	Met	Ala	Leu	Pro	Val	Pro	Pro	Ser	Pro	Phe	Phe Gln Gly
				320						325			330
	Ala	Glu	Arg	Trp	Pro	Pro	Gly	Val	Ala	Leu	Ser	Tyr	Leu His Ser
				335						340			345
15	Leu	Pro	Leu	Lys	Met	Gly	Gly	Asp	Gln	Arg	Ser	Met	Gly Leu Ala
				350						355			360
	Cys	Glu	Ser	Pro	Leu	Ala	Ala	Trp	Ser	Leu	Gly	Ile	Thr Pro Ala
				365						370			375
	Leu	Val	Leu	Gln	Met	Leu	Leu	Gly	Phe	Ile	Gly	Ala	Gly Pro Ser
				380						385			390
20	Arg	Ala	Gly	Pro	Leu	Thr	Leu	Pro	Ala	Trp	Leu	His	Ser Pro ***
				400						405			410
	Arg	Leu	Pro	Leu	Val	His	Pro	Phe	Ile	Glu	Arg	Pro	Ala Leu Leu
				415						420			425
25	Gln	Ser	Ser	Gly	Leu	Pro	Pro	Ala	Ala	Arg	Leu	Ser	Thr Arg Gly
				430						435			440
	Leu	Ser	***	Asp	Val	Gln	Gly	Pro	Arg	Pro	Ala	Gly	Thr Ala Ser
				445						450			455
	Pro	Asn	Leu	Gly	Pro	Trp	Lys	Ser	Pro	Pro	Pro	His	His *** Ser
				460						465			470
30	Ala	Leu	Thr	Leu	Gly	Phe	His	Gly	Pro	His	Ser	Thr	Ala Ser Pro
				475						480			485
	Thr	***	Ala	Cys	Asp	Leu	Gly	Thr	Lys	Gly	Gly	Val	Pro Arg Leu
				490						495			500
35	Leu	***	Leu	Ser	Arg	Gly	Ser	Gly	His	Val	Gln	Gly	Gly Ala Gly
				505						510			515
	Trp	Pro	Gly	Gly	Ser	Ala	His	Pro	Pro	Ala	Phe	Pro	Gln Gly Val
				520						525			530
	Leu	Arg	Ser	Lys	Ile	Leu	Glu	Gln	Ser	Asp	Pro	Ser	Pro Lys Ala
				535						540			545
40	Leu	Leu	Ser	Ala	Gly	Gln	Cys	Gln	Pro	Ile	Pro	Gly	His Leu Ala
				550						555			560
	Pro	Gly	Asp	Val	Gly	Pro	Xxx						
				565									

45

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 619 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

50

(ii) MOLECULE TYPE: amino acid (Translation of Contig 2535)

55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

60

Val	Phe	Tyr	Phe	Gly	Asn	Gly	Trp	Ile	Pro	Thr	Leu	Ile	Thr	Ala
1				5				10						15
Phe	Val	Leu	Ala	Thr	Ser	Gln	Ala	Gln	Ala	Gly	Trp	Leu	Gln	His

		20		25		30
	Asp Tyr Gly His	Leu Ser Val Tyr Arg Lys	Pro Lys Trp Asn His			
		35		40		45
5	Leu Val His Lys	Phe Val Ile Gly His Leu Lys Gly Ala Ser Ala				
		50		55		60
	Asn Trp Trp Asn	His Arg His Phe Gln His His Ala Lys Pro Asn				
		65		70		75
	Ile Phe His Lys	Asp Pro Asp Val Asn Met Leu His Val Phe Val				
		80		85		90
10	Leu Gly Glu Trp	Gln Pro Ile Glu Tyr Gly Lys Lys Lys Leu Lys				
		95		100		105
	Tyr Leu Pro Tyr	Asn His Gln His Glu Tyr Phe Phe Leu Ile Gly				
		110		115		120
15	Pro Pro Leu Leu	Ile Pro Met Tyr Phe Gln Tyr Gln Ile Ile Met				
		125		130		135
	Thr Met Ile Val	His Lys Asn Trp Val Asp Leu Ala Trp Ala Val				
		140		145		150
	Ser Tyr Tyr Ile	Arg Phe Phe Ile Thr Tyr Ile Pro Phe Tyr Gly				
		155		160		165
20	Ile Leu Gly Ala	Leu Leu Phe Leu Asn Phe Ile Arg Phe Leu Glu				
		170		175		180
	Ser His Trp Phe	Val Trp Val Thr Gln Met Asn His Ile Val Met				
		185		190		195
25	Glu Ile Asp Gln	Glu Ala Tyr Arg Asp Trp Phe Ser Ser Gln Leu				
		200		205		210
	Thr Ala Thr Cys	Asn Val Glu Gln Ser Phe Phe Asn Asp Trp Phe				
		215		220		225
	Ser Gly His Leu	Asn Phe Gln Ile Glu His His Leu Phe Pro Thr				
		230		235		240
30	Met Pro Arg His	Asn Leu His Lys Ile Ala Pro Leu Val Lys Ser				
		245		250		255
	Leu Cys Ala Lys	His Gly Ile Glu Tyr Gln Glu Lys Pro Leu Leu				
		260		265		270
35	Arg Ala Leu Leu	Asp Ile Ile Arg Ser Leu Lys Lys Ser Gly Lys				
		275		280		285
	Leu Trp Leu Asp	Ala Tyr Leu His Lys *** Ser His Ser Pro Arg				
		290		295		300
	Asp Thr Val Gly	Lys Gly Cys Arg Trp Gly Asp Gly Gln Arg Asn				
		305		310		315
40	Asp Gly Leu Leu	Phe *** Gly Val Ser Glu Arg Leu Val Tyr Ala				
		320		325		330
	Leu Leu Thr Asp	Pro Met Leu Asp Leu Ser Pro Phe Leu Leu Ser				
		335		340		345
45	Phe Phe Ser Ser	His Leu Pro His Ser Thr Leu Pro Ser Trp Asp				
		350		355		360
	Leu Pro Ser Leu	Ser Arg Gln Pro Ser Ala Met Ala Leu Pro Val				
		365		370		375
	Pro Pro Ser Pro	Phe Phe Gln Gly Ala Glu Arg Trp Pro Pro Gly				
		380		385		390
50	Val Ala Leu Ser	Tyr Leu His Ser Leu Pro Leu Lys Met Gly Gly				
		400		405		410
	Asp Gln Arg Ser	Met Gly Leu Ala Cys Glu Ser Pro Leu Ala Ala				
		415		420		425
55	Trp Ser Leu Gly	Ile Thr Pro Ala Leu Val Leu Gln Met Leu Leu				
		430		435		440
	Gly Phe Ile Gly	Ala Gly Pro Ser Arg Ala Gly Pro Leu Thr Leu				
		445		450		455
	Pro Ala Trp Leu	His Ser Pro *** Arg Leu Pro Leu Val His Pro				
		460		465		470
60	Phe Ile Glu Arg	Pro Ala Leu Leu Gln Ser Ser Gly Leu Pro Pro				
		475		480		485
	Ala Ala Arg Leu	Ser Thr Arg Gly Leu Ser *** Asp Val Gln Gly				

		490		495		500
	Pro Arg Pro Ala	Gly Thr Ala Ser Pro	Asn Leu Gly Pro Trp	Lys		
		505		510		515
5	Ser Pro Pro Pro	His His *** Ser Ala	Leu Thr Leu Gly Phe	His		
		520		525		530
	Gly Pro His Ser	Thr Ala Ser Pro Thr	*** Ala Cys Asp Leu	Gly		
		535		540		545
	Thr Lys Gly Gly	Val Pro Arg Leu Leu	*** Leu Ser Arg Gly	Ser		
		550		555		560
10	Gly His Val Gln	Gly Gly Ala Gly Trp	Pro Gly Gly Ser Ala	His		
		565		570		575
	Pro Pro Ala Phe	Pro Gln Gly Val Leu	Arg Ser Lys Ile Leu	Glu		
		580		585		590
	Gln Ser Asp Pro	Ser Pro Lys Ala Leu	Leu Ser Ala Gly Gln	Cys		
15		595		600		605
	Gln Pro Ile Pro	Gly His Leu Ala Pro	Gly Asp Val Gly Pro	Xxx		
		610		615		620

20

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 757 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: amino acid (Translation of Contig 253538a)

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

35	Gln Gly Pro Thr Pro Arg Tyr Phe Thr Trp Asp Glu Val Ala Gln	1	5	10	15
	Arg Ser Gly Cys Glu Glu Arg Trp Leu Val Ile Asp Arg Lys Val	20	25	30	
	Tyr Asn Ile Ser Phe Thr Arg Arg His Pro Gly Gly Ser Arg	35	40	45	
40	Val Ile Ser His Tyr Ala Gly Gln Asp Ala Thr Asp Pro Phe Val	50	55	60	
	Ala Phe His Ile Asn Lys Gly Leu Val Lys Lys Tyr Met Asn Ser	65	70	75	
45	Leu Leu Ile Gly Glu Leu Ser Pro Glu Gln Pro Ser Phe Glu Pro	80	85	90	
	Thr Lys Asn Lys Glu Leu Thr Asp Glu Phe Arg Glu Leu Arg Ala	95	100	105	
	Thr Val Glu Arg Met Gly Leu Met Lys Ala Asn His Val Phe Phe	110	115	120	
50	Leu Leu Tyr Leu Leu His Ile Leu Leu Leu Asp Gly Ala Ala Trp	125	130	135	
	Leu Thr Leu Trp Val Phe Gly Thr Ser Phe Leu Pro Phe Leu Leu	140	145	150	
55	Cys Ala Val Leu Leu Ser Ala Val Gln Gln Ala Gln Ala Gly Trp	155	160	165	
	Leu Gln His Asp Tyr Gly His Leu Ser Val Tyr Arg Lys Pro Lys	170	175	180	
	Trp Asn His Leu Val His Lys Phe Val Ile Gly His Leu Lys Gly	185	190	195	
60	Ala Ser Ala Asn Trp Trp Asn His Arg His Phe Gln His His Ala	200	205	210	
	Lys Pro Asn Ile Phe His Lys Asp Pro Asp Val Asn Met Leu His				

		215		220		225
	Val Phe Val Leu	Gly Glu Trp Gln Pro	Ile Glu Tyr Gly Lys Lys			
		230		235		240
5	Lys Leu Lys Tyr	Leu Pro Tyr Asn His	Gln His Glu Tyr Phe Phe			
		245		250		255
	Leu Ile Gly Pro	Pro Leu Leu Ile Pro	Met Tyr Phe Gln Tyr Gln			
		260		265		270
	Ile Ile Met Thr	Met Ile Val His Lys	Asn Trp Val Asp Leu Ala			
		275		280		285
10	Trp Ala Val Ser	Tyr Tyr Ile Arg Phe	Phe Ile Thr Tyr Ile Pro			
		290		295		300
	Phe Tyr Gly Ile	Leu Gly Ala Leu Leu	Phe Leu Asn Phe Ile Arg			
		305		310		315
	Phe Leu Glu Ser	His Trp Phe Val Trp	Val Thr Gln Met Asn His			
		320		325		330
15	Ile Val Met Glu	Ile Asp Gln Glu Ala	Tyr Arg Asp Trp Phe Ser			
		335		340		345
	Ser Gln Leu Thr	Ala Thr Cys Asn Val	Glu Gln Ser Phe Phe Asn			
		350		355		360
20	Asp Trp Phe Ser	Gly His Leu Asn Phe	Gln Ile Glu His His Leu			
		365		370		375
	Phe Pro Thr Met	Pro Arg His Asn Leu	His Lys Ile Ala Pro Leu			
		380		385		390
25	Val Lys Ser Leu	Cys Ala Lys His Gly	Ile Glu Tyr Gln Glu Lys			
		400		405		410
	Pro Leu Leu Arg	Ala Leu Leu Asp Ile	Ile Arg Ser Leu Lys Lys			
		415		420		425
	Ser Gly Lys Leu	Trp Leu Asp Ala Tyr	Leu His Lys *** Ser His			
		430		435		440
30	Ser Pro Arg Asp	Thr Val Gly Lys Gly	Cys Arg Trp Gly Asp Gly			
		445		450		455
	Gln Arg Asn Asp	Gly Leu Leu Phe ***	Gly Val Ser Glu Arg Leu			
		460		465		470
	Val Tyr Ala Leu	Leu Thr Asp Pro Met	Leu Asp Leu Ser Pro Phe			
		475		480		485
35	Leu Leu Ser Phe	Phe Ser Ser His Leu	Pro His Ser Thr Leu Pro			
		490		495		500
	Ser Trp Asp Leu	Pro Ser Leu Ser Arg	Gln Pro Ser Ala Met Ala			
		505		510		515
40	Leu Pro Val Pro	Pro Ser Pro Phe Phe	Gln Gly Ala Glu Arg Trp			
		520		525		530
	Pro Pro Gly Val	Ala Leu Ser Tyr Leu	His Ser Leu Pro Leu Lys			
		535		540		545
45	Met Gly Gly Asp	Gln Arg Ser Met Gly	Leu Ala Cys Glu Ser Pro			
		550		555		560
	Leu Ala Ala Trp	Ser Leu Gly Ile Thr	Pro Ala Leu Val Leu Gln			
		565		570		575
	Met Leu Leu Gly	Phe Ile Gly Ala Gly	Pro Ser Arg Ala Gly Pro			
		580		585		590
50	Leu Thr Leu Pro	Ala Trp Leu His Ser	Pro *** Arg Leu Pro Leu			
		595		600		605
	Val His Pro Phe	Ile Glu Arg Pro Ala	Leu Leu Gln Ser Ser Gly			
		610		615		620
55	Leu Pro Pro Ala	Ala Arg Leu Ser Thr	Arg Gly Leu Ser *** Asp			
		625		630		635
	Val Gln Gly Pro	Arg Pro Ala Gly Thr	Ala Ser Pro Asn Leu Gly			
		640		645		650
	Pro Trp Lys Ser	Pro Pro Pro His His	*** Ser Ala Leu Thr Leu			
		655		660		665
60	Gly Phe His Gly	Pro His Ser Thr Ala	Ser Pro Thr *** Ala Cys			
		670		675		680
	Asp Leu Gly Thr	Lys Gly Gly Val Pro	Arg Leu Leu *** Leu Ser			

		685		690		695
	Arg Gly Ser Gly	His Val Gln Gly Gly	Ala Gly Trp Pro Gly Gly			
		700		705		710
5	Ser Ala His Pro	Pro Ala Phe Pro Gln Gly Val Leu Arg Ser Lys				
		715		720		725
	Ile Leu Glu Gln	Ser Asp Pro Ser Pro Lys Ala Leu Leu Ser Ala				
		730		735		740
	Gly Gln Cys Gln	Pro Ile Pro Gly His Leu Ala Pro Gly Asp Val				
		745		750		755
10	Gly Pro Xxx					

(2) INFORMATION FOR SEQ ID NO:45:

15 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 746 nucleic acids
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

25	CGTATGTCAC TCCATTCCAA ACTCGTTCAT GGTATCATAA ATATCAACAC ATTTACGCTC	60
	CACTCCTCTA TGGTATTAC AACTCAAAT ATCGTACTCA AGATTGGGAA GCTTTTGTA	120
	AGGATGGTAA AAATGGTGCA ATTCGTGTTA GTGTCGCCAC AAATTTCGAT AAGGCCGCTT	180
	ACGTCATTGG TAAATTGTCT TTTGTTTCT TCCGTTTCAT CCTTCCACTC CGTTATCATA	240
30	GCTTTACAGA TTTAATTGT TATTCCTCA TTGCTGAATT CGTCTTGGT TGGTATCTCA	300
	CAATTAATTT CCAAGTTAGT CATGTCGCTG AAGATCTCAA ATTCTTTGCT ACCCCTGAAA	360
	GACCAGATGA ACCATCTCAA ATCAATGAAG ATTGGGCAAT CCTTCAACTT AAAACTACTC	420
	AAGATTATGG TCATGGTTCA CTCCTTGTA CCTTTTCTTA TGGTCTTTA AATCATCAAG	480
	TTGTTTCATCA TTTATTCCA TCAATTGCTC AAGATTCTA CCCACAACCT GTACCAATTG	540
35	TAAAAGAAGT TTGTAAAGAA CATAACATTA CTTACCACAT TAAACCAAAC TTCACTGAAG	600
	CTATTATGTC ACACATTAAT TACCTTTACA AAATGGGTAA TGATCCAGAT TATGTTAAAA	660
	AACCATTAGC CTCAAAAGAT GATTAAATGA AATACTTAA AAACCAATTA TTTACTTTTG	720
	ACAAACAGTA ATATTAATAA ATACAA	746

40 (2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 227 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

	Tyr Val Thr Pro Phe Gln Thr Arg Ser Trp Tyr His Lys Tyr Gln	
	1 5 10 15	
55	His Ile Tyr Ala Pro Leu Leu Tyr Gly Ile Tyr Thr Leu Lys Tyr	
	20 25 30	
	Arg Thr Gln Asp Trp Glu Ala Phe Val Lys Asp Gly Lys Asn Gly	
	35 40 45	
	Ala Ile Arg Val Ser Val Ala Thr Asn Phe Asp Lys Ala Ala Tyr	
	50 55 60	
60	Val Ile Gly Lys Leu Ser Phe Val Phe Phe Arg Phe Ile Leu Pro	
	65 70 75	
	Leu Arg Tyr His Ser Phe Thr Asp Leu Ile Cys Tyr Phe Leu Ile	
	80 85 90	
65	Ala Glu Phe Val Phe Gly Trp Tyr Leu Thr Ile Asn Phe Gln Val	
	95 100 105	

Ser His Val Ala Glu Asp Leu Lys Phe Phe Ala Thr Pro Glu Arg
 110 115 120
 Pro Asp Glu Pro Ser Gln Ile Asn Glu Asp Trp Ala Ile Leu Gln
 125 130 135
 5 Leu Lys Thr Thr Gln Asp Tyr Gly His Gly Ser Leu Leu Cys Thr
 140 145 150
 Phe Phe Ser Gly Ser Leu Asn His Gln Val Val His His Leu Phe
 155 160 165
 10 Pro Ser Ile Ala Gln Asp Phe Tyr Pro Gln Leu Val Pro Ile Val
 170 175 180
 Lys Glu Val Cys Lys Glu His Asn Ile Thr Tyr His Ile Lys Pro
 185 190 195
 Asn Phe Thr Glu Ala Ile Met Ser His Ile Asn Tyr Leu Tyr Lys
 200 205 210
 15 Met Gly Asn Asp Pro Asp Tyr Val Lys Lys Pro Leu Ala Ser Lys
 215 220 225
 Asp Asp ***

20 (2) INFORMATION FOR SEQ ID NO 47:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 494 nucleic acids
 (B) TYPE: nucleic acid
 25 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

TTTTGAAGG NTCCAAGTTN ACCACGGANT NGGCAAGTTN ACGGGGCGGA AANCGGTTTT 60
 35 CCCCCAAGC CTTTGTGCGA CTGGTTCTGT GGTGGCTTCC AGTACCAAGT CGACCACCAC 120
 TTATCCCCA GCCTGCCCCG ACACAATCTG GCCAAGACAC ACGCACTGGT CGAATCGTTC 180
 TGCAAGGAGT GGGGTGTCCA GTACCACGAA GCCGACCTCG TGGACGGGAC CATGGAAGTC 240
 TTGCACCATT TGGGCAGCGT GGCCGGCGAA TTCGTCGTGG ATTTTGTACG CGACGGACCC 300
 GCCATGTAAT CGTCGTTCGT GACGATGCAA GGGTTCACGC ACATCTACAC AACTCACTC 360
 40 ACACAAC TAGTAACTCGT ATAGAATTCTG GTGTGCACCT GGACCTTGTT TGACTGGTTG 420
 GGGATAGGGT AGGTAGGCGG ACGCGTGGGT CGNCCCCGGG AATTCTGTGA CCGGTACCTG 480
 GCCCGCGTNA AAGT 494

45 (2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 87 amino acids
 (B) TYPE: amino acid
 50 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

Phe Trp Lys Xxx Pro Ser Xxx Pro Arg Xxx Xxx Gln Val Xxx Gly
 1 5 10 15
 60 Ala Glu Xxx Gly Phe Pro Pro Lys Pro Phe Val Asp Trp Phe Cys
 20 25 30
 Gly Gly Phe Gln Tyr Gln Val Asp His His Leu Phe Pro Ser Leu
 35 40 45
 Pro Arg His Asn Leu Ala Lys Thr His Ala Leu Val Glu Ser Phe
 50 55 60
 65 Cys Lys Glu Trp Gly Val Gln Tyr His Glu Ala Asp Leu Val Asp
 65 70 75

Gly Thr Met Glu Val Leu His His Leu Gly Ser Val Ala Gly Glu
 65 70 75
 Phe Val Val Asp Phe Val Arg Asp Gly Pro Ala Met
 80 85

5

10

(2) INFORMATION FOR SEQ ID NO:49:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 520 nucleic acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

15

- (ii) MOLECULE TYPE: nucleic acid

20

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

25

30

GGATGGAGTT CGTCTGGATC GCTGTGCGCT ACGCGACGTG GTTTAAGCGT CATGGGTGCG 60
 CTTGGGTACA CGCCGGGGCA GTCGTTGGGC ATGTACTTGT GCGCCTTTGG TCTCGGCTGC 120
 ATTTACATTT TTCTGCAGTT CGCCGTAAGT CACACCCATT TGCCCGTGAG CAACCCGGAG 180
 GATCAGCTGC ATTGGCTCGA GTACGCGCGG ACCACACTGT GAACATCAGC ACCAAGTCGT 240
 GGTTCGTCAC ATGGTGGATG TCGAACCTCA ACTTTCAGAT CGAGCACCAC CTTTTCCTCCA 300
 CGGCGCCCCA GTTCCGTTTC AAGGAGATCA GCCCGCGCGT CGAGGCCCTC TTCAAGCGCC 360
 ACGGTCTCCC TTACTACGAC ATGCCCTACA CGAGCGCCGT CTCCACCACC TTTGCCAACC 420
 TCTACTCCGT CGGCCATTCC GTCGGCGACG CCAAGCGCGA CTAGCCTCTT TTCCTAGACC 480
 TTAATCCCC ACCCCACCCC ATGTTCTGTC TTCCTCCGCG 520

35

(2) INFORMATION FOR SEQ ID NO:50:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 153 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

40

- (ii) MOLECULE TYPE: peptide

45

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

50

55

60

65

Met Glu Phe Val Trp Ile Ala Val Arg Tyr Ala Thr Trp Phe Lys
 1 5 10 15
 Arg His Gly Cys Ala Trp Val His Ala Gly Ala Val Val Gly His
 20 25 30
 Val Leu Val Arg Leu Trp Ser Arg Leu His Leu His Phe Ser Ala
 35 40 45
 Val Arg Arg Lys Ser His Pro Phe Ala Arg Glu Gln Pro Gly Gly
 50 55 60
 Ser Ala Ala Leu Ala Arg Val Arg Ala Asp His Thr Val Asn Ile
 65 70 75
 Ser Thr Lys Ser Trp Phe Val Thr Trp Trp Met Ser Asn Leu Asn
 80 85 90
 Phe Gln Ile Glu His His Leu Phe Pro Thr Ala Pro Gln Phe Arg
 95 100 105
 Phe Lys Glu Ile Ser Pro Arg Val Glu Ala Leu Phe Lys Arg His
 110 115 120
 Gly Leu Pro Tyr Tyr Asp Met Pro Tyr Thr Ser Ala Val Ser Thr
 125 130 135
 Thr Phe Ala Asn Leu Tyr Ser Val Gly His Ser Val Gly Asp Ala

Lys Arg Asp 140 145 150

5

(2) INFORMATION FOR SEQ ID NO:51:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 429 nucleic acids
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: nucleic acid

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

ACGCGTCCGC CCACGCGTCC GCCGCGAGCA ACTCATCAAG GAAGGCTACT TTGACCCCTC 60
 GCTCCCGCAC ATGACGTACC GCGTGGTCGA GATTGTTGTT CTCTTCGTGC TTTCCCTTTG 120
 GCTGATGGGT CAGTCTTCAC CCCTCGCGCT CGCTCTCGGC ATTGTCGTCA GCGGCATCTC 180
 TCAGGGTCGC TCGGGCTGGG TAATGCATGA GATGGGCCAT GGGTCGTTCA CTGGTGTTCAT 240
 TTGGCTTGAC GACCGGTTGT GCGAGTTCTT TTACGGCGTT GGTTGTGGCA TGAGCGGTCA 300
 TTAGTGGAAA AACCAGCACA GCAAACACCA CGCAGCGCCA AACCGGCTCG AGCACGATGT 360
 AGATCTCAAC ACCTTGCCAT TGGTGGCCTT CAACGAGCGC GTCGTGCGCA AGGTCCGACC 420

25

30

(2) INFORMATION FOR SEQ ID NO:52:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 125 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

40

Arg Val Arg Pro Arg Val Arg Arg Glu Gln Leu Ile Lys Glu Gly
 1 5 10 15
 Tyr Phe Asp Pro Ser Leu Pro His Met Thr Tyr Arg Val Val Glu
 20 25 30
 Ile Val Val Leu Phe Val Leu Ser Phe Trp Leu Met Gly Gln Ser
 35 40 45
 Ser Pro Leu Ala Leu Ala Leu Gly Ile Val Val Ser Gly Ile Ser
 50 55 60
 Gln Gly Arg Cys Gly Trp Val Met His Glu Met Gly His Gly Ser
 65 70 75
 Phe Thr Gly Val Ile Trp Leu Asp Asp Arg Leu Cys Glu Phe Phe
 65 70 75
 Tyr Gly Val Gly Cys Gly Met Ser Gly His Tyr Trp Lys Asn Gln
 80 85 90
 His Ser Lys His His Ala Ala Pro Asn Arg Leu Glu His Asp Val
 95 100 105
 Asp Leu Asn Thr Leu Pro Leu Val Ala Phe Asn Glu Arg Val Val
 110 115 120
 Arg Lys Val Arg Pro
 125

60

What is claimed is:

1. A nucleic acid construct comprising:

One or more nucleotide sequences depicted in a SEQ ID NO: selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3 and SEQ ID NO:5,
5 wherein said one or more nucleotide sequences is linked to a heterologous nucleotide sequence.

2. A nucleic acid construct comprising:

One or more nucleotide sequences depicted in a SEQ ID NO: selected from
10 the group consisting of SEQ ID NO:1, SEQ ID NO:3 and SEQ ID NO:5,
wherein said one or more nucleotide sequences is operably associated with an expression control sequence functional in a plant cell.

3. The nucleic acid construct according to claim 2, wherein said nucleotide
15 sequence has an average A + T content of less than about 60%.

4. The nucleic acid construct according to claim 2, wherein said nucleotide
sequence is derived from a fungus.

20 5. The nucleic acid construct according to claim 4, wherein said fungus is of
the genus *Mortierella*.

6. The nucleic acid construct according to claim 5, wherein said fungus is of
the species *alpina*.

25 7. A nucleic acid construct comprising:

A nucleotide sequence which encodes a polypeptide comprising an amino
acid sequence depicted in SEQ ID NO:2, wherein said nucleotide sequence is

operably associated with a transcription or an expression control sequence function in a plant cell, wherein said nucleotide sequence encodes a functionally active polypeptide which desaturates a fatty acid molecule at carbon 6 from the carboxyl end of said fatty acid molecule.

5

8. A nucleic acid construct comprising:

A nucleotide sequence which encodes a polypeptide comprising an amino acid sequence depicted in SEQ ID NO:4, wherein said nucleotide sequence is operably associated with a transcription or an expression control sequence functional in a plant cell, wherein said nucleotide sequence encodes a functionally active polypeptide which desaturates a fatty acid molecule at carbon 12 from the carboxyl end of said fatty acid molecule.

10

9. A nucleic acid construct comprising:

A nucleotide sequence which encodes a polypeptide comprising an amino acid sequence depicted in SEQ ID NO:6, wherein said nucleotide sequence is operably associated with a transcription or an expression control sequence function in a plant cell, wherein said nucleotide sequence encodes a functionally active polypeptide which desaturates a fatty acid molecule at carbon 5 from the carboxyl end of said fatty acid molecule.

15

20

10. A nucleic acid construct comprising:

at least one nucleotide sequence which encodes a functionally active desaturase having an amino acid sequence depicted in a SEQ ID NO: selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:6, wherein said nucleotide sequence is operably associated with a promoter functional in a plant cell.

25

11. The nucleic acid construct according to claim 10, wherein said plant cell is a seed cell.

5 12. The nucleic acid construct according to claim 11, wherein said seed cell is an embryo cell.

13. A recombinant plant cell comprising:

10 At least one copy of a DNA sequence which encodes at least one functionally active *Mortierella alpina* fatty acid desaturase which results in the production of a polyunsaturated fatty acid, wherein said fatty acid desaturase has an amino acid sequence as depicted in a SEQ ID NO: selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, and SEQ ID NO:6, wherein said cell was transformed with a vector comprising said DNA sequence, and
15 wherein said DNA sequence is operably associated with an expression control sequence.

14. The recombinant plant cell of claim 13, wherein said polyunsaturated fatty acid is selected from the group consisting of LA, ARA, GLA, DGLA, SDA and EPA.
20

15. The recombinant plant cell of claim 13, wherein said recombinant plant cell is enriched in a fatty acid selected from the group consisting of 18:1, 18:2, 18:3 and 18:4.

25 16. The recombinant plant cell of claim 15, wherein said plant cell is selected from the group consisting of *Brassica*, soybean, safflower, corn, flax, and sunflower.

17. The recombinant plant cell according to claim 16, wherein said expression control sequence is endogenous to said plant cell.

18. One or more plant oils expressed by said recombinant plant cell of claim 16.

5

19. A method for obtaining altered long chain polyunsaturated fatty acid biosynthesis comprising the steps of:

growing a plant having cells which contain a transgene encoding a transgene expression product which desaturates a fatty acid molecule at carbon
10 5 from the carboxyl end of said fatty acid molecule, wherein said transgene is operably associated with an expression control sequence, under conditions whereby said transgene is expressed, whereby long chain polyunsaturated fatty acid biosynthesis in said cells is altered.

15 20. A method for obtaining altered long chain polyunsaturated fatty acid biosynthesis comprising the steps of:

growing a plant having cells which contain one or more transgenes, derived from a fungus or algae, which encodes a transgene expression product which desaturates a fatty acid molecule at a carbon selected from the group
20 consisting of carbon 5, carbon 6 and carbon 12 from the carboxyl end of said fatty acid molecule, wherein said one or more transgenes is operably associated with an expression control sequence, under conditions whereby said one or more transgenes is expressed, whereby long chain polyunsaturated fatty acid biosynthesis in said cells is altered.

25

21. The method according to claims 19 or 20, wherein said long chain polyunsaturated fatty acid is selected from the group consisting of LA, ARA, GLA, DGLA, SDA and EPA.

22. A plant oil or fraction thereof produced according to the method of claims 19 or 20.
- 5 23. A method of treating or preventing malnutrition comprising administering said plant oil of claim 22 to a patient in need of said treatment or prevention in an amount sufficient to effect said treatment or prevention.
24. A pharmaceutical composition comprising said plant oil or fraction of claim 22 and a pharmaceutically acceptable carrier.
- 10 25. The pharmaceutical composition of claim 24, wherein said pharmaceutical composition is in the form of a solid or a liquid.
- 15 26. The pharmaceutical composition of claim 25, wherein said pharmaceutical composition is in a capsule or tablet form.
- 20 27. The pharmaceutical composition of claim 24 further comprising at least one nutrient selected from the group consisting of a vitamin, a mineral, a carbohydrate, a sugar, an amino acid, a free fatty acid, a phospholipid, an antioxidant, and a phenolic compound.
28. A nutritional formula comprising said plant oil or fraction thereof of claim 22.
- 25 29. The nutritional formula of claim 28, wherein said nutritional formula is selected from the group consisting of an infant formula, a dietary supplement, and a dietary substitute.

30. The nutritional formula of claim 29, wherein said infant formula, dietary supplement or dietary supplement is in the form of a liquid or a solid.

31. An infant formula comprising said plant oil or fraction thereof of claim 22.

5

32. The infant formula of claim 31 further comprising at least one macronutrient selected from the group consisting of coconut oil, soy oil, canola oil, mono- and diglycerides, glucose, edible lactose, electrodialysed whey, electrodialysed skim milk, milk whey, soy protein, and other protein hydrolysates.

10

33. The infant formula of claim 32 further comprising at least one vitamin selected from the group consisting of Vitamins A, C, D, E, and B complex; and at least one mineral selected from the group consisting of calcium, magnesium, zinc, manganese, sodium, potassium, phosphorus, copper, chloride, iodine, selenium, and iron.

15

34. A dietary supplement comprising said plant oil or fraction thereof of claim 22.

20

35. The dietary supplement of claim 34 further comprising at least one macronutrient selected from the group consisting of coconut oil, soy oil, canola oil, mono- and diglycerides, glucose, edible lactose, electrodialysed whey, electrodialysed skim milk, milk whey, soy protein, and other protein hydrolysates.

25

36. The dietary supplement of claim 35 further comprising at least one vitamin selected from the group consisting of Vitamins A, C, D, E, and B complex; and at least one mineral selected from the group consisting of calcium,

magnesium, zinc, manganese, sodium, potassium, phosphorus, copper, chloride, iodine, selenium, and iron.

5 37. The dietary supplement of claim 34 or claim 36, wherein said dietary supplement is administered to a human or an animal.

38. A dietary substitute comprising said plant oil or fraction thereof of claim 22.

10 39. The dietary substitute of claim 38 further comprising at least one macronutrient selected from the group consisting of coconut oil, soy oil, canola oil, mono- and diglycerides, glucose, edible lactose, electrodialysed whey, electrodialysed skim milk, milk whey, soy protein, and other protein hydrolysates.

15 40. The dietary substitute of claim 39 further comprising at least one vitamin selected from the group consisting of Vitamins A, C, D, E, and B complex; and at least one mineral selected from the group consisting of calcium, magnesium, zinc, manganese, sodium, potassium, phosphorus, copper, chloride, iodine, selenium, and iron.

20 41. The dietary substitute of claim 38 or claim 40, wherein said dietary substitute is administered to a human or animal.

25 42. A method of treating a patient having a condition caused by insufficient intake or production of polyunsaturated fatty acids comprising administering to said patient said dietary substitute of claim 38 or said dietary supplement of claim 34 in an amount sufficient to effect said treatment.

43. The method of claim 42, wherein said dietary substitute or said dietary supplement is administered enterally or parenterally.

44. A cosmetic comprising said plant oil or fraction thereof of claim 22.

5

45. The cosmetic of claim 44, wherein said cosmetic is applied topically.

46. The pharmaceutical composition of claim 24, wherein said pharmaceutical composition is administered to a human or an animal.

10

47. An animal feed comprising said plant oil or fraction thereof of claim 22.

48. An isolated nucleotide sequence comprising the nucleotide sequence selected from the group consisting of SEQ ID NO:38 - SEQ ID NO:44 wherein said nucleotide sequence is expressed in a plant cell.

15

49. The method of claim 20 wherein said fungus is *Mortierella species*.

50. The method of claim 49 wherein said fungus is *Mortierella alpina*.

20

51. An isolated nucleotide sequence selected from the group consisting of SEQ ID NO:49 - SEQ ID NO:50 wherein said sequence is expressed in a plant cell.

1/20

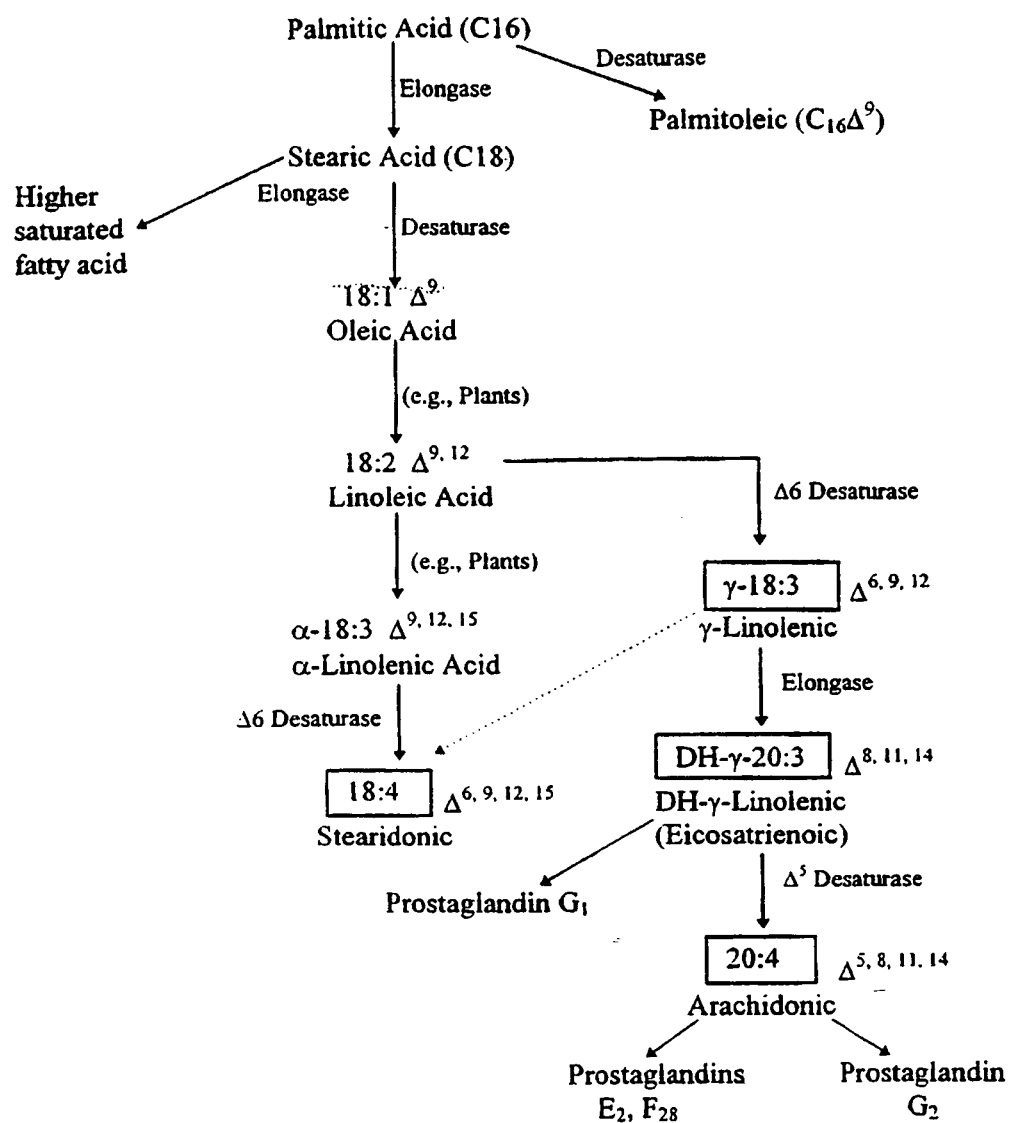


FIG. 1

PUFA PATHWAYS

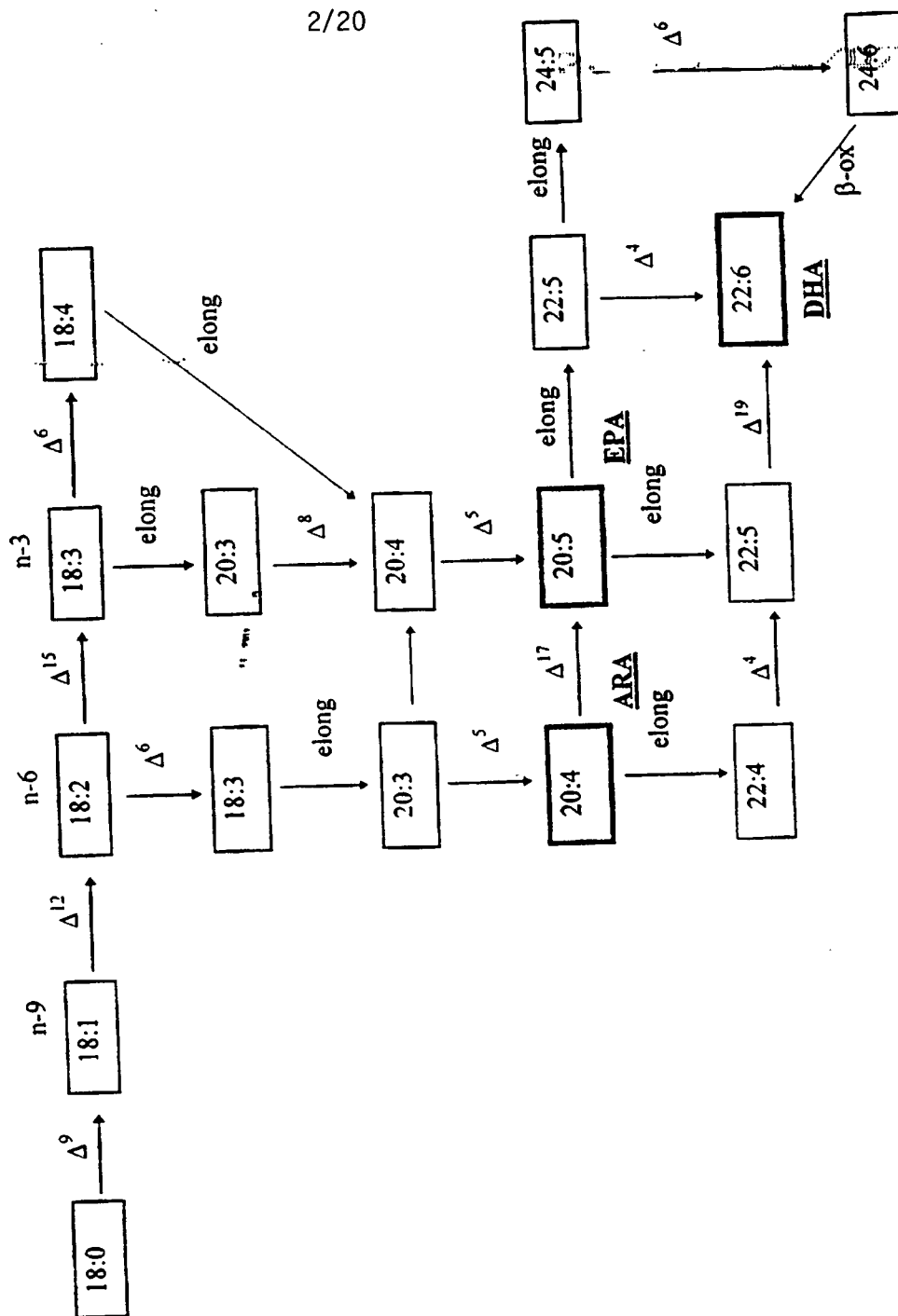


FIG. 2

60 *
 CGACACTCCT TCCTTCTTCT CACCCGTCCT AGTCCCTTC AACCCCTTC TTGACAAAG
 ACAACAAACC ATG GCT GCT CCC AGT GTG AGG ACG TTT ACT GGG GCC GAG
 Met Ala Ala Pro Ser Val Arg Thr Phe Thr Arg Ala Glu
 120 *
 GTT TTG AAT GCC GAG GCT CTG AAT GAG GGC AAG AAG GAT GCC GAG GCA
 Val Leu Asn Ala Glu Ala Leu Asn Glu Gly Lys Lys Asp Ala Glu Ala
 180 *
 CCC TTC TTG ATG ATC ATC GAC AAC AAG GTG TAC GAT GTC CGC GAG TTC
 Pro Phe Leu Met Ile Ile Asp Asn Lys Val Tyr Asp Val Arg Glu Phe
 240 *
 GTC CCT GAT CAT CCC GGT GGA AGT GTG ATT CTC ACG CAC GTT GGC AAG
 Val Pro Asp His Pro Gly Gly Ser Val Ile Leu Thr His Val Gly Lys
 300 *
 GAC GGC ACT GAC GTC TTT GAC ACT TTT CAC CCC GAG GCT GCT TGG GAG
 Asp Gly Thr Asp Val Phe Asp Thr Phe His Pro Glu Ala Ala Tyr Glu
 ACT CTT GCC AAC TTT TAC GTT GGT GAT ATT GAC GAG AGC GAC CGC GAT
 Thr Leu Ala Asn Phe Tyr Val Gly Asp Ile Asp Glu Ser Asp Arg Asp
 360 *
 ATC AAG AAT GAT GAC TTT GCG GCC GAG GTC CGC AAG CTG CGT ACC TTG
 Ile Lys Asn Asp Asp Phe Ala Ala Glu Val Arg Lys Leu Arg Thr Leu

FIG. 3A

420 *
 TTC CAG TCT CTT GGT TAC TAC GAT TCT TCC AAG GCA TAC TAC GCC TTC
 Phe Gln Ser Leu Gly Tyr Tyr Asp Ser Ser Lys Ala Tyr Tyr Ala Phe
 480 *
 AAG GTC TCG TTC AAC CTC TGC ATC TGG GGT TTG TCG ACG GTC ATT GTG
 Lys Val Ser Phe Asn Leu Cys Ile Trp Gly Leu Ser Thr Val Ile Val
 540 *
 GCC AAG TGG GGC CAG ACC TCG ACC CTC GCC AAC GTG CTC TCG GCT GCG
 Ala Lys Trp Gly Gln Thr Ser Thr Leu Ala Asn Val Leu Ser Ala Ala
 CTT TTG GGT CTG TTC TGG CAG CAG TGC GGA TGG TTG GCT CAC GAC TTT
 Leu Leu Gly Leu Phe Trp Gln Gln Cys Gly Trp Leu Ala His Asp Phe
 600 *
 TTG CAT CAC CAG GTC TTC CAG GAC CGT TTC TGG GGT GAT CTT TTC GGC
 Leu His His Gln Val Phe Gln Asp Arg Phe Trp Gly Asp Leu Phe Gly
 660 *
 GCC TTC TTG GGA GGT GTC TGC CAG GGC TTC TCG TCC TCG TGG TGG AAG
 Ala Phe Leu Gly Gly Val Cys Gln Gly Phe Ser Ser Ser Trp Trp Lys
 720 *
 GAC AAG CAC AAC ACT CAC CAC GCC GCC CCC AAC GTC CAC GGC GAG GAT
 Asp Lys His Asn Thr His His Ala Ala Pro Asn Val His Gly Glu Asp
 780 *

FIG. 3B

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CCC GAC ATT GAC ACC CAC CCT CTG TTG ACC TGG AGT GAG CAT GCG TTG
 Pro Asp Ile Asp Thr His Pro Leu Leu, Thr Trp Ser Glu His Ala Leu

 GAG ATG TTC TCG GAT GTC CCA GAT GAG GAG CTG ACC CGC ATG TGG TCG
 Glu Met Phe Ser Asp Val Pro Asp Glu Glu Leu Thr Arg Met Trp Ser

 840 *
 CGT TTC ATG GTC CTG AAC CAG ACC TGG TTT TAC TTC CCC ATT CTC TCG
 Arg Phe Met Val Leu Asn Gln Thr Trp Phe Tyr Phe Pro Ile Leu Ser

 900 *
 TTT GCC CGT CTC TCC TGG TGC CTC CAG TCC ATT CTC TTT GTG CTG CCT
 Phe Ala Arg Leu Ser Trp Cys Leu Gln Ser Ile Leu Phe Val Leu Pro

 960 *
 AAC GGT CAG GCC CAC AAG CCC TCG GGC GCG CGT GTG CCC ATC TCG TTG
 Asn Gly Gln Ala His Lys Pro Ser Gly Ala Arg Val Pro Ile Ser Leu

 1020 *
 GTC GAG CAG CTG TCG CTT GCG ATG CAC TGG ACC TGG TAC CTC GCC ACC
 Val Glu Gln Leu Ser Leu Ala Met His Trp Thr Trp Tyr Leu Ala Thr

 ATG TTC CTG TTC ATC AAG GAT CCC GTC AAC ATG CTG GTG TAC TTT TTG
 Met Phe Leu Phe Ile Lys Asp Pro Val Asn Met Leu Val Tyr Phe Leu

 1080 *
 GTG TCG CAG GCG GTG TGC GGA AAC TTG TGG GCG ATC GTG TTC TCG CTC
 Val Ser Gln Ala Val Cys Gly Asn Leu Leu Ala Ile Val Phe Ser Leu

FIG. 3C

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1140 *
AAC CAC AAC GGT ATG CCT GTG ATC TCG AAG GAG GAG GCG GTC GAT ATG
Asn His Asn Gly Met Pro Val Ile Ser Lys Glu Glu Ala Val Asp Met

1200 *
GAT TTC TTC ACG AAG CAG ATC ATC ACG GGT CGT GAT GTC CAC CCG GGT
Asp Phe Phe Thr Lys Gln Ile Ile Thr Gly Arg Asp Val His Pro Gly

1260 *
CTA TTT GCC AAC TGG TTC ACG GGT GGA TTG AAC TAT CAG ATC GAG CAC
Leu Phe Ala Asn Trp Phe Thr Gly Gly Leu Asn Tyr Gln Ile Glu His

1320 *
CAC TTG TTC CCT TCG ATG CCT CGC CAC AAC TTT TCA AAG ATC CAG CCT
His Leu Phe Pro Ser Met Pro Arg His Asn Phe Ser Lys Ile Gln Pro

1380 *
GCT GTC GAG ACC CTG TGC AAA AAG TAC AAT GTC CGA TAC CAC ACC ACC
Ala Val Glu Thr Leu Cys Lys Lys Tyr Asn Val Arg Tyr His Thr Thr

1440 *
GGT ATG ATC GAG GGA ACT GCA GAG GTC TTT AGC CGT CTG AAC GAG GTC
Gly Met Ile Glu Gly Thr Ala Glu Val Phe Ser Arg Leu Asn Glu Val

1440 *
TCC AAG GCT GCC TCC AAG ATG GGT AAG GCG CAG TAAAAAAA AAACAAGGAC
Ser Lys Ala Ala Ser Lys Met Gly Lys Ala Gln

FIG. 3D

1500 *
GTTTTTTTTC GCCAGTGCCT GTGCCTGTGC CTGCTTCCCT TGTCAAGTCG AGCGTTTCTG
1560 *
GAAAGGATCG TTCAGTGCAG TATCATCATTT CTCCTTTTAC CCCCCGCTCA TATCTCATTC
ATTCTCTTTA TTAAACAACCT TGTTCCTCCCC TTCACCG

FIG. 3E

60
 38
 24
 3
 0
 1
 0
 119
 96
 83
 3
 1
 0
 174
 140
 104
 3
 0
 1
 0
 229
 104
 88
 90
 23
 105
 349
 104
 252
 83
 143
 355
 104
 252
 125
 131
 87
 143

EYKJATLIGUICGLRSLRAYZAKVSEMLCINGLSIVIVAKNGOISTLANVLSAALLGL
 VILT-TLATVARMSCGULIGVLACSVXPKNOITAGLLGL
 GLIGVLACTSVFQNOIADLLGL
 CXX-
 F42006
 W20140
 R05219
 H53753
 FHOCCGLAHJNCLHNOYFORREHNDLFCAILGCVC
 IHSATIGXOSCHYVIMSKSMNK-FAOLESCHMC
 LHSASAVIGESCUYVIMSHRSYUR-FAOLUSCHNLCTISIAMWMTTHAAHMLACINSLDY
 T42006
 W20140
 R05219
 H53753
 DPRIIDRUPLLITHSEJALEHESDYPDEELTRHNS--REMVLNOTMEFYPLLSFARLSM
 GPHLOHIDVIABTK--FE3SLTSRFYUBKLTGCPVARBLQSIOHFTUDVMCFGBHL
 CLCSILEVLEPUGOAHKPSGARPISLVEOLSIAM--IHHTHYLAETHETFKDPVHHLV
 FIOTFDLFSERE--VBONALHEAGILV--FWTF--PLLVSCLPUMPERF
 --HFAGILV--FWTF--PLLVSCLPUMPERF
 --PATEVGGLAMHIIFYVRFLTYVPLLCGLKAPFG
 --
 --RUCANAGGTRLAYHLEVCHQHTDL--LMAASFYSRFFLSYSPFYGATGTL
 TELYSOAVCGUJIAIVLSLHUGHPVISREFAYDDDFEIKOIIIGRDYHPGLEAHNFEGG
 FEVFTSFVTALQWHIFTEHHFAADVTV-GPTTCSOWFERQAAGTIIDISCRTSYMDHFFGG
 HEVTCGFVTAIONIQEELHHFAADVTV-GPTTCSOWHELEOAAGTIIDISCRTSYMDHFFGG
 LEVFVAFLESBWFWVWTOHJJ--IPMHIDMDRHMDHVSTOLOATCNHVKSAFENDHWESGH
 L2VAVRVULESHHFWMITOMHJ--IPKEIGHKHRRHASSQLAATCNCELEFIIDHESGH
 LNLOIEHHJFPSSHCNHHSKLQEA.V.E.I.LCKKYNVNYHTGMIEGTAEVPSALNEVSKRAS
 IIOIQLEHHJPPRPFCHLRLREVSPVGGRGEOBKXHLX
 LRIOICMHLFPSTMPRNHNHXVADELVOSELCAHMGITEYOSRPL
 LHYOICMHLFPSTMPRNHNENRCHXYWNCAREHLPYLVDODITYGKXHLHLOBKKNHAELVQ
 LUTOLECHHLFPSTMPRNJURYRXVADLUNAKAFGRHGLHJEV
 KHGKAQ
 H40524
 ATTS4123
 32-5
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 R05219
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 --AKBA

FIG. 4

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60
GTCCCCGTGC GCTGTGGGCA CACCCCATCC TCCCTCGCTC CCTCTGGGTT TGTCCTTGGC
120
CCACCGTATC TCCTCCACCC TCCGAGACGA CTGCACTGT AATCAGGAAC CGACAAATAC
180
ACGATTTCTT TTTACTCAGC ACCAACTCAA AATCCTCAAC CGCAACCCCTT TTTCAGG ATG
Met
GCA CCT CCC AAC ACT ATC GAT GCC GGT TTG ACC CAG CGT CAT ATC AGC
Ala Pro Pro Asn Thr Ile Asp Ala Gly Leu Thr Gln Arg His Ile Ser
240
ACC TCG GCC CCA AAC TCG GCC AAG CCT GCC TTC GAG CGC AAC TAC CAG
Thr Ser Ala Pro Asn Ser Ala Lys Pro Ala Phe Glu Arg Asn Tyr Gln
300
CTC CCC GAG TTC ACC ATC AAG GAG ATC CGA GAG TGC ATC CCT GCC CAC
Leu Pro Glu Phe Thr Ile Lys Glu Ile Arg Glu Cys Ile Pro Ala His
360
TGC TTT GAG CGC TCC GGT CTC CGT GGT CTC TGC CAC GTT GCC ATC GAT
Cys Phe Glu Arg Ser Gly Leu Arg Gly Leu Cys His Val Ala Ile Asp
420
CTG ACT TGG GCG TCG CTC TTG TTC CTG GCT GCG ACC CAG ATC GAC AAG
Leu Thr Trp Ala Ser Leu Leu Phe Leu Ala Ala Thr Gln Ile Asp Lys
TTT GAG AAT CCC TTG ATC CGC TAT TTG GCC TGG CCT GTT TAC TGG ATC
Phe Glu Asn Pro Leu Ile Arg Tyr Leu Ala Trp Pro Val Tyr Trp Ile

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FIG. 5A

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480
 ATG CAG GGT ATT GTC TGC ACC GGT GTC TGG GTG CTG GCT CAC GAG TGT
 Met Gln Gly Ile Val Cys Thr Gly Val Trp Val Leu Ala His Glu Cys

540
 GGT CAT CAG TCC TTC TCG ACC TCC AAG ACC CTC AAC ACA GGT GGT
 Gly His Gln Ser Phe Ser Thr Ser Lys Thr Leu Asn Thr Val Gly

600
 TGG ATC TTG CAC TCG ATG CTC TTG GTC CCC TAC CAC TCC TGG AGA ATC
 Trp Ile Leu His Ser Met Leu Leu Val Pro Tyr His Ser Trp Arg Ile

660
 TCG CAC TCG AAG CAC CAC AAG GCC ACT GGC CAT ATG ACC AAG GAC CAG
 Ser His Ser Lys His His Lys Ala Thr Gly His Met Thr Lys Asp Gln

720
 GTC TTT GTG CCC AAG ACC CGC TCC CAG GTT GGC TTG CCT CCC AAG GAG
 Val Phe Val Pro Lys Thr Arg Ser Gln Val Gly Leu Pro Pro Lys Glu

780
 AAC GCT GCT GCT GCC GGT CAG GAG GAG GAC ATG TCC GTG CAC CTG GAT
 Asn Ala Ala Ala Ala Val Gln Glu Glu Asp Met Ser Val His Leu Asp

840
 GAG CAG GCT CCC ATT GTG ACT TTG TTC TGG ATG GTG ATC CAG TTC TTG
 Glu Glu Ala Pro Ile Val Thr Leu Phe Trp Met Val Ile Gln Phe Leu

840
 TTC GGA TGG CCC GCG TAC CTG ATT ATG AAC GCC TCT GGC CAA GAC TAC
 Phe Gly Trp Pro Ala Tyr Leu Ile Met Asn Ala Ser Gly Gln Asp Tyr

FIG. 5B

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900 *
GGC CGC TGG ACC TCG CAC TTC CAC ACG TAC TCG CCC ATC TTT GAG CGC
Gly Arg Trp Thr Ser His Phe His Thr Tyr Ser Pro Ile Phe Glu Pro

CGC AAC TTT TTC GAC ATT ATT ATC TCG GAC CTC GGT GTG TTG GCT GCC
Arg Asn Phe Phe Asp Ile Ile Ile Ser Asp Leu Gly Val Leu Ala Ala

960 *
CTC GGT GCC CTG ATC TAT GCC TCC ATG ATG CAG TTG TCG CTC TTG ACC GTC
Leu Gly Ala Leu Ile Tyr Ala Ser Met Gln Leu Ser Leu Leu Thr Val

1020 *
TACC AAG TAC TAT ATT GTC CCC TAC CTC TTT GTC AAC TTT TGG TTG GTC
Thr Lys Tyr Tyr Ile Val Pro Tyr Leu Phe Val Asn Phe Trp Leu Val

1080 *
CTG ATC ACC TTC TTG CAG CAC ACC GAT CCC AAG CTG CCC CAT TAC CGC
Leu Ile Thr Phe Phe Leu Gln His Thr Asp Pro Lys Leu Pro His Tyr Arg

1140 *
GAG GGT GCC TGG AAT TTC CAG CGT GGA GCT CTT TGC ACC GTT GAC CGC
Glu Gly Ala Trp Asn Phe Gln Arg Gly Ala Leu Cys Thr Val Asp Arg

TCG TTT GGC AAG TTC TTG GAC CAT ATG TTC CAC GGC ATT GTC CAC ACC
Ser Phe Gly Lys Phe Leu Asp His Met Phe His Gly Ile Val His Thr

1200 *
CAT GTG GCC CAT CAC TTG TTC TCG CAA ATG CCG TTC TAC CAT GCT GAG
His Val Ala His His Leu Phe Ser Gln Met Pro Phe Tyr His Ala Glu

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FIG. 5C

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1260 *
GAA GCT ACC TAT CAT CTC AAG AAA CTG CTG GGA GAG TAC TAT GTG TAC
Glu Ala Thr Tyr His Leu Lys Lys Leu Gly Glu Tyr Tyr Val Tyr
1320 *
GAC CCA TCC CCG ATC GTC GTT GCG GTC TGG AGG TCG TTC CGT GAG TGC
Asp Pro Ser Pro Ile Val Val Ala Val Trp Arg Ser Phe Arg Glu Cys
1380 *
CGA TTC GTG GAG GAT CAG GGA GAC GTG GTC TTT TTT AAG AAG TAAAAA
Arg Phe Val Glu Asp Gln Gly Asp Val Val Phe Phe Lys Lys
1440 *
AAAAGACAAT GGACCACACA CAACCTTGTC TCTACAGACC TACGTATCAT GTAGCCATAC
CACTTCATAA AAGAACATGA GCTCTAGAGG CGTGTCTATC GCGCCTCC

FIG. 5D

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FIG. 6

10	20	30	40	50	60
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LHHTYTNIAG ADPDVSTSEP DVRRKPNQK WFNHINQHM FVPFLYGLLA FKVRIQDINI					
70	80	90	100	110	120
					*
LYFVKTNDAI RVNPISTWHT VMFWGGKAFF VWYRLIVPLQ YLPLGKVLLL FTVADMVSSY					
130	140	150	160	170	180
					*
WLALTFQANY VVEEVQWPLP DENGIIQKDW AAMQVETTQD YAHDSHLWTS ITGSLNYQXV					
HHLFFPH					

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FIG. 7A

GCTTCCTCCA GTTCATCCTC CATTTCGCCA CCTGCATTCT TTACGACCGT TAAGCAAG
 60 *
 ATG GGA ACG GAC CAA GGA AAA ACC TTC ACC TGG GAA GAG CTG GCG GCC
 Met Gly Thr Asp Gln Gly Lys Thr Phe Thr Trp Glu Glu Leu Ala Ala
 120 *
 CAT AAC ACC AAG GAC GAC CTA CTC TTG GCC ATC CGC GGC AGG GTG TAC
 His Asn Thr Lys Asp Asp Leu Leu Leu Ala Ile Arg Gly Arg Val Tyr
 180 *
 GAT GTC ACA AAG TTC TTG AGC CGC CAT CCT GGT GGA GTG GAC ACT CTC
 Asp Val Thr Lys Phe Leu Ser Arg His Pro Gly Gly Val Asp Thr Leu
 240 *
 CTG CTC GGA GCT GGC CGA GAT GTT ACT CCG GTC TTT GAG ATG TAT CAC
 Leu Leu Gly Ala Gly Arg Asp Val Thr Pro Val Phe Glu Met Tyr His
 GCG TTT GGG GCT GCA GAT GCC ATT ATG AAG AAG TAC TAT GTC GGT ACA
 Ala Phe Gly Ala Ala Asp Ala Ile Met Lys Lys Tyr Tyr Val Gly Thr
 300 *
 CTG GTC TCG AAT GAG CTG CCC ATC TTC CCG GAG CCA ACG GTG TTC CAC
 Leu Val Ser Asn Glu Leu Pro Ile Phe Pro Glu Pro Thr Val Phe His
 360 *
 AAA ACC ATC AAG ACG AGA GTC GAG GGC TAC TTT ACG GAT CGG AAC ATT
 Lys Thr Ile Lys Thr Arg Val Glu Gly Tyr Phe Thr Asp Arg Asn Ile

FIG. 7B

420 *
 GAT CCC AAG AAT AGA CCA GAG ATC TGG GGA CGA TAC GCT CTT ATC TTT
 Asp Pro Lys Asn Arg Pro Glu Ile Trp Gly Arg Tyr Ala Leu Ile Phe
 480 *
 GGA TCC TTG ATC GCT TCC TAC TAC GCG CAG CTC TTT GTG CCT TTC GTT
 Gly Ser Leu Ile Ala Ser Tyr Tyr Ala Gln Leu Phe Val Phe Val
 GTC GAA CGC ACA TGG CTT CAG GTG GTG TTT GCA ATC ATC ATG GGA TTT
 Val Glu Arg Thr Trp Leu Gln Val Val Phe Ala Ile Ile Met Gly Phe
 540 *
 GCG TGC GCA CAA GTC GGA CTC AAC CCT CTT CAT GAT GCG TCT CAC TTT
 Ala Cys Ala Gln Val Gly Leu Asn Pro Leu His Asp Ala Ser His Phe
 600 *
 TCA GTG ACC CAC AAC CCC ACT GTC TGG AAG ATT CTG GGA GCC ACG CAC
 Ser Val Thr His Asn Pro Thr Val Trp Lys Ile Leu Gly Ala Thr His
 660 *
 GAC TTT TTC AAC GGA GCA TCG TAC CTG GTG TGG ATG TAC CAA CAT ATG
 Asp Phe Phe Asn Gly Ala Ser Tyr Leu Val Trp Met Tyr Gln His Met
 720 *
 CTC GGC CAT CAC CCC TAC ACC AAC ATT GCT GGA GCA GAT CCC GAC GTG
 Leu Gly His His Pro Tyr Thr Asn Ile Ala Gly Ala Asp Pro Asp Val

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FIG. 7C

TCG ACG TCT GAG CCC GAT GTT CGT ATC AAG CCC AAC CAA AAG TGG
 Ser Thr Ser Glu Pro Asp Val Arg Arg Ile Lys Pro Asn Gln Lys Trp
 780 *

TTT GTC AAC CAC ATC AAC CAG CAC ATG TTT GTT CCT TTC CTG TAC GGA
 Phe Val Asn His Ile Asn Gln His Met Phe Val Pro Phe Leu Tyr Gly
 840 *

CTG CTG GCG TTC AAG GTG CGC ATT CAG GAC ATC AAC ATT TTG TAC TTT.
 Leu Leu Ala Phe Lys Val Arg Ile Gln Asp Ile Asn Ile Leu Tyr Phe
 900 *

GTC AAG ACC AAT GAC GCT ATT CGT GTC AAT CCC ATC TCG ACA TGG CAC
 Val Lys Thr Asn Asp Ala Ile Arg Val Asn Pro Ile Ser Thr Trp His
 960 *

ACT GTG ATG TTC TGG GGC AAG GCT TTC TTT GTC TGG TAT CGC CTG
 Thr Val Met Phe Trp Gly Gly Lys Ala Phe Phe Val Trp Tyr Arg Leu
 1020 *

ATT GTT CCC CTG CAG TAT CTG CCC CTG GGC AAG GTG CTG CTC TTG TTC
 Ile Val Pro Leu Gln Tyr Leu Pro Leu Gly Lys Val Leu Leu Phe
 1020 *

ACG GTC GCG GAC ATG GTG TCG TCT TAC TGG CTG GCG CTG ACC TTC CAG
 Thr Val Ala Asp Met Val Ser Ser Tyr Trp Leu Ala Leu Thr Phe Gln

FIG. 7D

1080
 GCG AAC CAC GTT GTT GAG GAA GTT CAG TGG CCG TTG CCT GAC GAG AAC
 Ala Asn His Val Val Glu Glu Val Gln Trp Pro Leu Pro Asp Glu Asn

1140
 GGG ATC ATC CAA AAG GAC TGG GCA GCT ATG CAG GTC GAG ACT ACG CAG
 Gly Ile Ile Gln Lys Asp Trp Ala Ala Met Gln Val Glu Thr Thr Gln

1200
 GAT TAC GCA CAC GAT TCG CAC CTC TGG ACC AGC ATC ACT GGC AGC TTG.
 Asp Tyr Ala Ala His Asp Ser His Leu Trp Thr Ser Ile Thr Gly Ser Leu

1260
 AAC TAC CAG GCT GTG CAC CAT CTG TTC CCG AAC GTG TCG CAG CAC CAT
 Asn Tyr Gln Ala Val His His Leu Phe Pro Asn Val Ser Gln His His

1320
 TAT CCC GAT ATT CTG GCC ATC ATC AAG AAC ACC TGC AGC GAG TAC AAG
 Tyr Pro Asp Ile Leu Ala Ile Ile Lys Asn Thr Cys Ser Glu Tyr Lys

1380
 GTT CCA TAC CTT GTC AAG GAT ACG TTT TGG CAA GCA TTT GCT TCA CAT
 Val Pro Tyr Leu Val Lys Asp Thr Phe Trp Gln Ala Phe Ala Ser His

1440
 TTG GAG CAC TTG CGT GTT CTT GGA CTC CGT CCC AAG GAA GAG TAGA
 Leu Glu His Leu Arg Val Leu Gly Leu Arg Pro Lys Glu Glu

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 QAAGTGGACA TTCTCTATGA AGA

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HA29	10	20	30	40	50	60	70
HA524	11	21	31	41	51	61	71
BA206	12	22	32	42	52	62	72
Sy680106	13	23	33	43	53	63	73
Sp106	14	24	34	44	54	64	74
HA29	80	90	100	110	120	130	140
HA524	81	91	101	111	121	131	141
BA206	82	92	102	112	122	132	142
Sy680106	83	93	103	113	123	133	143
Sp106	84	94	104	114	124	134	144
HA29	150	160	170	180	190	200	210
HA524	151	161	171	181	191	201	211
BA206	152	162	172	182	192	202	212
Sy680106	153	163	173	183	193	203	213
Sp106	154	164	174	184	194	204	214
HA29	220	230	240	250	260	270	280
HA524	221	231	241	251	261	271	281
BA206	222	232	242	252	262	272	282
Sy680106	223	233	243	253	263	273	283
Sp106	224	234	244	254	264	274	284
HA29	290	300	310	320	330	340	350
HA524	291	301	311	321	331	341	351
BA206	292	302	312	322	332	342	352
Sy680106	293	303	313	323	333	343	353
Sp106	294	304	314	324	334	344	354
HA29	360	370	380	390	400	410	420
HA524	361	371	381	391	401	411	421
BA206	362	372	382	392	402	412	422
Sy680106	363	373	383	393	403	413	423
Sp106	364	374	384	394	404	414	424
HA29	430	440	450	460	470	480	490
HA524	431	441	451	461	471	481	491
BA206	432	442	452	462	472	482	492
Sy680106	433	443	453	463	473	483	493
Sp106	434	444	454	464	474	484	494

FIG. 8

Figure 9

20/20

FastA Match of ma524 and contig 253538a

SCORES Init1: 231 Initn: 499 Opt: 401
 Smith-Waterman score: 620; 27.3% identity in 455 aa overlap

```

      10      20      30      40      50      59
ma524gcg.pep MAAAPSVRTFTTAEVLNAEALNEGKKDAEAPFLMIIDNKVYDVREFVPDHPGGSVILTH-
      | : | | | | : : : : : : | | | : | : | | | : : |
253538a      QGPTPRYFTWDEV-----AQRSGCEERWLVIDRKVYNISEFTRRHPPGGSRVISHY
      10      20      30      40      50

      60      70      80      90      100     110
ma524gcg.pep VGKDGTDVFDTFHPEAAW--ETLANFYVGDIDE---SDRDIKNDDFAAEVRKLRTLTFQSL
      | : | | | | : : | : : : : : | : | : | : | : | : | : |
253538a      AGQDATDPFVAFHINKGLVKKYNMSLLIGELSPEQPSFEPTKNKELTDEFREL RATVERM
      60      70      80      90      100     110

      120     130     140     150     160     170
ma524gcg.pep GYYDSSKAYYAFKVSFNLCIWLSTVIVAKWGQTSTLANVLSAALLGLFWQCGWLAHDF
      | : : : : : : : | : : : : : | : | : | : | : | : | : |
253538a      GLMKANHVFLLYLLHILLLDGAAWLTWVFG-TSFLPFLLCVLLSAVQAQAGWLQHDY
      120     130     140     150     160

      180     190     200     210     220     230
ma524gcg.pep LHHQVFQDRFWDLFGAFLGGVCQGFSSSWWKDKHNTTHAAPNVHGEDPDIDTHPLLTWS
      | : | : : | : | : | : | : | : | : | : | : | : | : |
253538a      GHLSVYRKPKWNHLVHKFVIGHLGASANWNNHRHFQHHAKPNIFHKDPVN---ML---
      170     180     190     200     210     220

      240     250     260     270     280     290
ma524gcg.pep EHALEMFSVDPDEELTRMWSRFVNLQTFWYFPILS---FARLSWCLQSILFVLPNGQAH
      | : : : : | : : : : | : | : : : : : : : : : | : : : : |
253538a      -HVF-VLGEWQPIEYGKKLKYLPYNHQHEYFFLIGPPLLIPMYFQYQIIMIMI---VH
      230     240     250     260     270

      300     310     320     330     340     349
ma524gcg.pep KPSGARVPISLVEQLSLAMHWTWYLATMFLFIK--DPVNMLVYFLVSQAVCGNLLAIVFS
      | : : | : : : : | : : : : | : : : : | : : : : | : : : : | :
253538a      K-----NWVDLAWAVSYIIRFFITYIPFYGILGALLFLNFIREFLESHWFWVWVTO
      280     290     300     310     320

      350     360     370     380     390     400     409
ma524gcg.pep LNHNGMPVISKEEAVDMDEFTKQIITGRDVHPGLFANWFTGGLNYQIEHHLFSPMPRHNH
      : | | : : : | : | : : : : : : : : : | : | : | : | : | : | : |
253538a      MNHIVMEI--DQEAYR-DWFSSQLTATCNVEQSFFNDWFSGHLNFQIEHHLFPTMPRHNH
      330     340     350     360     370     380

      410     420     430     440     450
ma524gcg.pep SKIQPAVETLCKKYNVRYHTTGMIEGTAEVFSRLNEVSKAASKMGAQX
      | | | : : | : : : : : : : : : : : : : : : | : : : |
253538a      HKIAPLVKSLCAKHGIEYQEKPLLRLALLDIIRSLKKSGKLWLDAYLHKX
      390     400     410     420     430

```

Figure 10

INTERNATIONAL SEARCH REPORT

In Application No
PCT/US 98/07421

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/53 C12N15/82 C12N5/10 C12P7/64 C11B1/00
A61K31/20 A23L1/30 A23K1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12P C11B A61K A23L A23K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 06712 A (RHONE POULENC AGROCHIMIE) 15 April 1993 cited in the application see the whole document ---	20-22
X	WO 94 18337 A (MONSANTO CO ;UNIV MICHIGAN (US); GIBSON SUSAN IRMA (US); KISHORE G) 18 August 1994 * see the whole document, esp. claims 8-10 *	20-47
X	WO 96 21022 A (RHONE POULENC AGROCHIMIE) 11 July 1996 cited in the application * see the whole document, esp. p. 2 1.3-21 * --- -/--	20-47



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search

21 August 1998

Date of mailing of the international search report

03/09/1998

Name and mailing address of the ISA

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Authorized officer

Kania, T

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/07421

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 561 569 A (LUBRIZOL CORP) 22 September 1993 cited in the application see the whole document ----	20-47
A	COVELLO P. ET AL.: "Functional expression of the extraplastidial Arabidopsis thaliana oleate desaturase gene (FAD2) in Saccharomyces cerevisiae" PLANT PHYSIOLOGY, vol. 111, no. 1, May 1996, pages 223-226, XP002075211 see the whole document ----	1-51
A	WO 94 11516 A (DU PONT ;LIGHTNER JONATHAN EDWARD (US); OKULEY JOHN JOSEPH (US)) 26 May 1994 cited in the application see the whole document ----	1-51
T	WO 97 30582 A (CARNEGIE INST OF WASHINGTON ;MONSANTO COMPANY INC (US); BROUN PIER) 28 August 1997 see the whole document -----	1-51

INTERNATIONAL SEARCH REPORT

ern application No.

PCT/US 98/07421

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 23, 42, 43
are directed to a method of treatment of the human/animal
body, the search has been carried out and based on the alleged
effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/ US 98 /07421

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (group of) inventions in this international application, as follows:

1. Claims 1-47, 49,50

Nucleic acid constructs comprising delta-5, delta-6, or delta-12 desaturases according to SEQ ID NO: 1,3,5, derived from the fungus *Mortierella alpina*.
Recombinant plant cells comprising said constructs.
Methods for obtaining altered long chain polyunsaturated fatty acid biosynthesis using plants comprising delta-5, delta-6, or delta-12 desaturases, or combinations thereof, derived from fungi or algae.
Plant oils derived from said plants and their use for therapeutical, nutritional, and cosmetical purposes, as well as products derived therefrom.

2. Claim : 48

An isolated sequence comprising the nucleotide sequence selected from the group of SEQ ID NO: 38-44, wherein said nucleotide is expressed in a plant cells.

3. Claim : 51

An isolated nucleotide sequence selected from the group consisting of SEQ ID NO: 49-50, wherein said sequence is expressed in a plant cell.

INTERNATIONAL SEARCH REPORT

Information on patent family members

In Application No

PCT/US 98/07421

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/07421

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9730582 A	28-08-1997	AU 2050497 A	10-09-1997



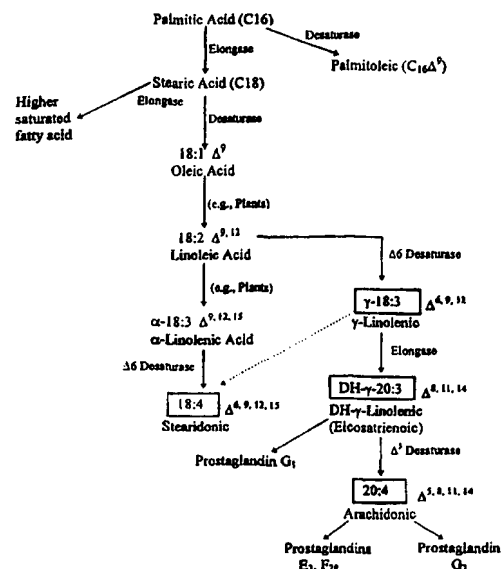
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/53, 15/82, 5/10, C12P 7/64, C11B 1/00, A61K 31/20, A23L 1/30, A23K 1/00		A1	(11) International Publication Number: WO 98/46764
			(43) International Publication Date: 22 October 1998 (22.10.98)
(21) International Application Number: PCT/US98/07421		ABBOTT LABORATORIES [US/US]; 100 Abbott Park Road, Abbott Park, IL 60064-3500 (US).	
(22) International Filing Date: 10 April 1998 (10.04.98)		(72) Inventors; and	
(30) Priority Data:		(75) Inventors/Applicants (for US only): KNUTZON, Deborah [US/US]; 6110 Rockhurst Way, Granite Bay, CA 95746 (US). MUKERJI, Pradip [US/US]; 1069 Arcaro Drive, Gahanna, OH 43230 (US). HUANG, Yung-Sheng [CA/US]; 2462 Danvers Court, Upper Arlington, OH 43220 (US). THURMOND, Jennifer [US/US]; 3702 Adirondack, Columbus, OH 43231 (US). CHAUDHARY, Sunita [IN/US]; 3419 Woodbine Place, Pearland, TX 77584 (US). LEONARD, Amanda, Eun-Yeong [US/US]; 581 Shadewood Court, Gahanna, OH 43230 (US).	
08/833,610 11 April 1997 (11.04.97) US			
08/834,033 11 April 1997 (11.04.97) US			
08/834,655 11 April 1997 (11.04.97) US			
08/956,985 24 October 1997 (24.10.97) US			
(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications		(74) Agents: WARD, Michael, R. et al.; Limbach & Limbach L.L.P., 2001 Ferry Building, San Francisco, CA 94111-4262 (US).	
US 08/834,655 (CIP)			
Filed on 11 April 1997 (11.04.97)			
US 08/833,610 (CIP)			
Filed on 11 April 1997 (11.04.97)			
US 08/834,033 (CIP)			
Filed on 11 April 1997 (11.04.97)			
US 08/956,985 (CIP)			
Filed on 24 October 1997 (24.10.97)			
(71) Applicants (for all designated States except US): CALGENE LLC [US/US]; 1920 Fifth Street, Davis, CA 95616 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
		Published	
		With international search report.	
		Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.	

(54) Title: METHODS AND COMPOSITIONS FOR SYNTHESIS OF LONG CHAIN POLYUNSATURATED FATTY ACIDS IN PLANTS

(57) Abstract

The present invention relates to compositions and methods for preparing polyunsaturated long chain fatty acids in plants, plant parts and plant cells, such as leaves, roots, fruits and seeds. Nucleic acid sequences and constructs encoding fatty acid desaturases, including Δ^5 -desaturases, Δ^6 -desaturases and Δ^{12} -desaturases, are used to generate transgenic plants, plant parts and cells which contain and express one or more transgenes encoding one or more desaturases. Expression of the desaturases with different substrate specificities in the plant system permit the large scale production of polyunsaturated long chain fatty acids such as docosahexaenoic acid, eicosapentaenoic acid, α -linolenic acid, gamma-linolenic acid, arachidonic acid and the like for modification of the fatty acid profile of plants, plant parts and tissues. Manipulation of the fatty acid profiles allows for the production of commercial quantities of novel plant oils and products.



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--	----	--

(21) International Application Number: PCT/US98/07421

(22) International Filing Date: 10 April 1998 (10.04.98)

(30) Priority Data:

08/833,610	11 April 1997 (11.04.97)	US
08/834,033	11 April 1997 (11.04.97)	US
08/834,655	11 April 1997 (11.04.97)	US
08/956,985	24 October 1997 (24.10.97)	US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications

US	08/834,655 (CIP)
Filed on	11 April 1997 (11.04.97)
US	08/833,610 (CIP)
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US	08/834,033 (CIP)
Filed on	11 April 1997 (11.04.97)
US	08/956,985 (CIP)
Filed on	24 October 1997 (24.10.97)

(71) Applicants (for all designated States except US): CALGENE LLC [US/US]; 1920 Fifth Street, Davis, CA 95616 (US).

ABBOTT LABORATORIES [US/US]; 100 Abbott Park Road, Abbott Park, IL 60064-3500 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): KNUTZON, Deborah [US/US]; 6110 Rockhurst Way, Granite Bay, CA 95746 (US). MUKERJI, Pradip [US/US]; 1069 Arcaro Drive, Gahanna, OH 43230 (US). HUANG, Yung-Sheng [CA/US]; 2462 Danvers Court, Upper Arlington, OH 43220 (US). THURMOND, Jennifer [US/US]; 3702 Adirondack, Columbus, OH 43231 (US). CHAUDHARY, Sunita [IN/US]; 3419 Woodbine Place, Pearland, TX 77584 (US). LEONARD, Amanda, Eun-Yeong [US/US]; 581 Shadewood Court, Gahanna, OH 43230 (US).

(74) Agents: WARD, Michael, R. et al.; Limbach & Limbach L.L.P., 2001 Ferry Building, San Francisco, CA 94111-4262 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

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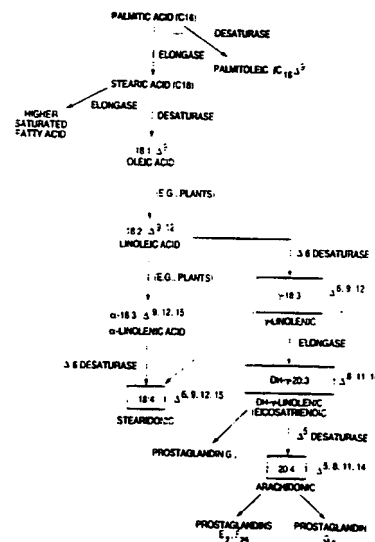
With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

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(57) Abstract

The present invention relates to compositions and methods for preparing polyunsaturated long chain fatty acids in plants, plant parts and plant cells, such as leaves, roots, fruits and seeds. Nucleic acid sequences and constructs encoding fatty acid desaturases, including $\Delta 5$ -desaturases, $\Delta 6$ -desaturases and $\Delta 12$ -desaturases, are used to generate transgenic plants, plant parts and cells which contain and express one or more transgenes encoding one or more desaturases. Expression of the desaturases with different substrate specificities in the plant system permit the large scale production of polyunsaturated long chain fatty acids such as docosahexaenoic acid, eicosapentaenoic acid, α -linolenic acid, gamma-linolenic acid, arachidonic acid and the like for modification of the fatty acid profile of plants, plant parts and tissues. Manipulation of the fatty acid profiles allows for the production of commercial quantities of novel plant oils and products.



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METHODS AND COMPOSITIONS FOR SYNTHESIS OF LONG CHAIN POLYUNSATURATED FATTY ACIDS IN PLANTS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of USSN 08/834,655, filed
5 April 11, 1997, and a continuation in part of USSN 08/833,610, filed April 11,
1997, USSN 08/834,033 filed April 11, 1997 and USSN 08/956,985 filed
October 24, 1997 which disclosures are incorporated herein by reference.

INTRODUCTION

Field of the Invention

10 This invention relates to modulating levels of enzymes and/or enzyme
components capable of altering the production of long chain polyunsaturated
fatty acids (PUFAS) in a host plant. The invention is exemplified by the
production of PUFAS in plants.

Background

15 Two main families of polyunsaturated fatty acids (PUFAs) are the $\omega 3$
fatty acids, exemplified by arachidonic acid, and the $\omega 6$ fatty acids, exemplified
by eicosapentaenoic acid. PUFAs are important components of the plasma
membrane of the cell, where they may be found in such forms as phospholipids.
PUFAs also serve as precursors to other molecules of importance in human
20 beings and animals, including the prostacyclins, leukotrienes and
prostaglandins. PUFAs are necessary for proper development, particularly in
the developing infant brain, and for tissue formation and repair.

Four major long chain PUFAs of importance include docosahexaenoic
acid (DHA) and eicosapentaenoic acid (EPA), which are primarily found in
25 different types of fish oil, gamma-linolenic acid (GLA), which is found in the
seeds of a number of plants, including evening primrose (*Oenothera biennis*),
borage (*Borago officinalis*) and black currants (*Ribes nigrum*), and stearidonic
acid (SDA), which is found in marine oils and plant seeds. Both GLA and
another important long chain PUFA, arachidonic acid (ARA), are found in

filamentous fungi. ARA can be purified from animal tissues including liver and adrenal gland.

For DHA, a number of sources exist for commercial production including a variety of marine organisms, oils obtained from cold water marine fish, and egg yolk fractions. For ARA, microorganisms including the genera *Mortierella*, *Entomophthora*, *Phytium* and *Porphyridium* can be used for commercial production. Commercial sources of SDA include the genera *Trichodesma* and *Echium*. Commercial sources of GLA include evening primrose, black currants and borage. However, there are several disadvantages associated with commercial production of PUFAs from natural sources. Natural sources of PUFAs, such as animals and plants, tend to have highly heterogeneous oil compositions. The oils obtained from these sources therefore can require extensive purification to separate out one or more desired PUFAs or to produce an oil which is enriched in one or more PUFA. Natural sources also are subject to uncontrollable fluctuations in availability. Fish stocks may undergo natural variation or may be depleted by overfishing. Fish oils have unpleasant tastes and odors, which may be impossible to economically separate from the desired product, and can render such products unacceptable as food supplements. Animal oils, and particularly fish oils, can accumulate environmental pollutants. Weather and disease can cause fluctuation in yields from both fish and plant sources. Cropland available for production of alternate oil-producing crops is subject to competition from the steady expansion of human populations and the associated increased need for food production on the remaining arable land. Crops which do produce PUFAs, such as borage, have not been adapted to commercial growth and may not perform well in monoculture. Growth of such crops is thus not economically competitive where more profitable and better established crops can be grown. Large scale fermentation of organisms such as *Mortierella* is also expensive. Natural animal tissues contain low amounts of ARA and are difficult to process. Microorganisms such as *Porphyridium* and *Mortierella* are difficult to cultivate on a commercial scale.

Dietary supplements and pharmaceutical formulations containing PUFAs can retain the disadvantages of the PUFA source. Supplements such as fish oil capsules can contain low levels of the particular desired component and thus require large dosages. High dosages result in ingestion of high levels of undesired components, including contaminants. Care must be taken in providing fatty acid supplements, as overaddition may result in suppression of endogenous biosynthetic pathways and lead to competition with other necessary fatty acids in various lipid fractions *in vivo*, leading to undesirable results. For example, Eskimos having a diet high in ω 3 fatty acids have an increased tendency to bleed (U.S. Pat. No. 4,874,603). Unpleasant tastes and odors of the supplements can make such regimens undesirable, and may inhibit compliance by the patient.

A number of enzymes are involved in PUFA biosynthesis. Linoleic acid (LA, 18:2 Δ 9, 12) is produced from oleic acid (18:1 Δ 9) by a Δ 12-desaturase. GLA (18:3 Δ 6, 9, 12) is produced from linoleic acid (LA, 18:2 Δ 9, 12) by a Δ 6-desaturase. ARA (20:4 Δ 5, 8, 11, 14) production from DGLA (20:3 Δ 8, 11, 14) is catalyzed by a Δ 5-desaturase. However, animals cannot desaturate beyond the Δ 9 position and therefore cannot convert oleic acid (18:1 Δ 9) into linoleic acid (18:2 Δ 9, 12). Likewise, α -linolenic acid (ALA, 18:3 Δ 9, 12, 15) cannot be synthesized by mammals. Other eukaryotes, including fungi and plants, have enzymes which desaturate at positions Δ 21 and Δ 15. The major poly-unsaturated fatty acids of animals therefore are either derived from diet and/or from desaturation and elongation of linoleic acid (18:2 Δ 9, 12) or α -linolenic acid (18:3 Δ 9, 12, 15).

Poly-unsaturated fatty acids are considered to be useful for nutritional, pharmaceutical, industrial, and other purposes. An expansive supply of poly-unsaturated fatty acids from natural sources and from chemical synthesis are not sufficient for commercial needs. Therefore it is of interest to obtain genetic material involved in PUFA biosynthesis from species that naturally produce these fatty acids and to express the isolated material alone or in combination in

a heterologous system which can be manipulated to allow production of commercial quantities of PUFAS.

The present invention is further directed to formulas, dietary supplements or dietary supplements in the form of a liquid or a solid containing
5 the long chain fatty acids of the invention. These formulas and supplements may be administered to a human or an animal.

The formulas and supplements of the invention may further comprise at least one macronutrient selected from the group consisting of coconut oil, soy oil, canola oil, mono- and diglycerides, glucose, edible lactose, electro dialysed
10 whey, electro dialysed skim milk, milk whey, soy protein, and other protein hydrolysates.

The formulas of the present invention may further include at least one vitamin selected from the group consisting of Vitamins A, C, D, E, and B complex; and at least one mineral selected from the group consisting of
15 calcium, magnesium, zinc, manganese, sodium, potassium, phosphorus, copper, chloride, iodine, selenium, and iron.

The present invention is further directed to a method of treating a patient having a condition caused by insufficient intake or production of polyunsaturated fatty acids comprising administering to the patient a dietary substitute of the
20 invention in an amount sufficient to effect treatment of the patient.

The present invention is further directed to cosmetic and pharmaceutical compositions of the material of the invention.

The present invention is further directed to transgenic oils in pharmaceutically acceptable carriers. The present invention is further directed
25 to nutritional supplements, cosmetic agents and infant formulae containing transgenic oils.

The present invention is further directed to a method for obtaining altered long chain polyunsaturated fatty acid biosynthesis comprising the steps of: growing a microbe having cells which contain a transgene which encodes a

transgene expression product which desaturates a fatty acid molecule at carbon 5,5 or 12 from the carboxyl end of said fatty acid molecule, wherein the transgene is operably associated with an expression control sequence, under conditions whereby the transgene is expressed, whereby long chain
5 polyunsaturated fatty acid biosynthesis in the cells is altered.

The present invention is further directed toward pharmaceutical compositions comprising at least one nutrient selected from the group consisting of a vitamin, a mineral, a carbohydrate, a sugar, an amino acid, a free fatty acid, a phospholipid, an antioxidant, and a phenolic compound.

10 Relevant Literature

Production of gamma-linolenic acid by a $\Delta 6$ -desaturase is described in USPN 5,552,306 and USPN 5,614,393. Production of 8, 11-eicosadienoic acid using *Mortierella alpina* is disclosed in USPN 5,376,541. Production of docosahexaenoic acid by dinoflagellates is described in USPN 5,407,957.
15 Cloning of a $\Delta 6$ -desaturase from borage is described in PCT publication WO 96/21022. Cloning of $\Delta 9$ -desaturases is described in the published patent applications PCT WO 91/13972, EP 0 550 162 A1, EP 0 561 569 A2, EP 0 644 263 A2, and EP 0 736 598 A1, and in USPN 5,057,419. Cloning of $\Delta 12$ -desaturases from various organisms is described in PCT publication WO
20 94/11516 and USPN 5,443,974. Cloning of $\Delta 15$ -desaturases from various organisms is described in PCT publication WO 93/11245. A $\Delta 6$ palmitoyl-acyl carrier protein desaturase from *Thumbergia alata* and its expression in *E. coli* is described in USPN 5,614,400. Expression of a soybean stearyl-ACP desaturase in transgenic soybean embryos using a 35S promoter is disclosed in USPN
25 5,443,974.

SUMMARY OF THE INVENTION

Novel compositions and methods are provided for preparation of polyunsaturated long chain fatty acids and desaturases in plants and plant cells. The methods involve growing a host plant cell of interest transformed with an
30 expression cassette functional in a host plant cell, the expression cassette

comprising a transcriptional and translational initiation regulatory region, joined in reading frame 5' to a DNA sequence encoding a desaturase polypeptide capable of modulating the production of PUFAs. Expression of the desaturase polypeptide provides for an alteration in the PUFA profile of host plant cells as a result of altered concentrations of enzymes involved in PUFA biosynthesis. Of particular interest is the selective control of PUFA production in plant tissues and/or plant parts such as leaves, roots, fruits and seeds. The invention finds use for example in the large scale production of DHA, EPA, ARA, and GLA and for modification of the fatty acid profile of edible plant tissues and/or plant parts.

The present invention further includes a purified nucleotide sequence or polypeptide sequence that is substantially related or homologous to the nucleotide and peptide sequences presented in SEQ ID NO:1 - SEQ ID NO:52. The present invention is further directed to methods of using the sequences presented in SEQ ID NO:1 to SEQ ID NO:40 as probes to identify related sequences, as components of expression systems and as components of systems useful for producing transgenic oil.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows possible pathways for the synthesis of arachidonic acid (20:4 Δ 5, 8, 11, 14) and stearidonic acid (18:4 Δ 6, 9, 12, 15) from palmitic acid (C_{16}) from a variety of organisms, including algae, *Mortierella* and humans. These PUFAs can serve as precursors to other molecules important for humans and other animals, including prostacyclins, leukotrienes, and prostaglandins, some of which are shown.

Figure 2 shows possible pathways for production of PUFAs in addition to ARA, including EPA and DHA, again compiled from a variety of organisms.

Figure 3A-E shows the DNA sequence (SEQ ID NO:1) of the *Mortierella alpina* Δ 6 desaturase and the deduced amino acid sequence (SEQ ID NO:2).

Figure 4 shows an alignment of the *Mortierella alpina* $\Delta 6$ desaturase amino acid sequence with other $\Delta 6$ desaturases and related sequences (SEQ ID NOS:7, 8, 9, 10, 11, 12 and 13).

Figure 5A-D shows the DNA sequence of the *Mortierella alpina* $\Delta 12$ desaturase (SEQ ID NO:3) and the deduced amino acid sequence (SEQ ID NO:4)

Figure 6 shows the deduced amino acid sequence (SEQ ID NO:14) of the PCR fragment (see Example 1).

Figure 7A-D shows the DNA sequence of the *Mortierella alpina* $\Delta 5$ desaturase (SEQ ID NO:5).

Figure 8 shows alignments of the protein sequence of the $\Delta 5$ desaturase (SEQ ID NO:6) with $\Delta 6$ desaturases and related sequences (SEQ ID NOS:15, 16, 17, 18).

Figure 9 shows alignments of the protein sequence of the Ma 29 and contig 253538a.

Figure 10 shows alignments of the protein sequence of Ma 524 and contig 253538a.

BRIEF DESCRIPTION OF THE SEQUENCE LISTINGS

SEQ ID NO:1 shows the DNA sequence of the *Mortierella alpina* $\Delta 6$ desaturase.

SEQ ID NO:2 shows the amino acid sequence of the *Mortierella alpina* $\Delta 6$ desaturase.

SEQ ID NO:3 shows the DNA sequence of the *Mortierella alpina* $\Delta 12$ desaturase.

SEQ ID NO:4 shows the amino acid sequence of the *Mortierella alpina* $\Delta 12$ desaturase.

SEQ ID NO:5 shows the DNA sequence of the *Mortierella alpina* $\Delta 5$ desaturase.

SEQ ID NO:6 shows the amino acid sequence *Mortierella alpina* $\Delta 5$ desaturase.

5 SEQ ID NO:7 - SEQ ID NO:13 show amino acid sequences that relate to *Mortierella alpina* $\Delta 6$ desaturase.

SEQ ID NO:14 shows an amino acid sequence of a PCR fragment of Example 1.

10 SEQ ID NO:15 - SEQ ID NO:18 show amino acid sequences that relate to *Mortierella alpina* $\Delta 5$ and $\Delta 6$ desaturases.

SEQ ID NO:19 - SEQ ID NO:30 show PCR primer sequences.

SEQ ID NO:31 - SEQ ID NO:37 show human nucleotide sequences.

SEQ ID NO:38 - SEQ ID NO:44 show human peptide sequences.

15 SEQ ID NO:45 - SEQ ID NO:46 show the nucleotide and amino acid sequence of a *Dictyostelium discoideum* desaturase.

SEQ ID NO:47 - SEQ ID NO:50 show the nucleotide and deduced amino acid sequence of a *Schizochytrium* cDNA clone.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

20 In order to ensure a complete understanding of the invention, the following definitions are provided:

$\Delta 5$ -Desaturase: $\Delta 5$ desaturase is an enzyme which introduces a double bond between carbons 5 and 6 from the carboxyl end of a fatty acid molecule.

$\Delta 6$ -Desaturase: $\Delta 6$ -desaturase is an enzyme which introduces a double bond between carbons 6 and 7 from the carboxyl end of a fatty acid molecule.

25 **$\Delta 9$ -Desaturase:** $\Delta 9$ -desaturase is an enzyme which introduces a double bond between carbons 9 and 10 from the carboxyl end of a fatty acid molecule.

Δ 12-Desaturase: Δ 12-desaturase is an enzyme which introduces a double bond between carbons 12 and 13 from the carboxyl end of a fatty acid molecule.

Fatty Acids: Fatty acids are a class of compounds containing a long hydrocarbon chain and a terminal carboxylate group. Fatty acids include the following:

Fatty Acid		
12:0	lauric acid	
16:0	palmitic acid	
16:1	palmitoleic acid	
18:0	stearic acid	
18:1	oleic acid	Δ 9-18:1
18:2 Δ 5,9	taxoleic acid	Δ 5,9-18:2
18:2 Δ 6,9	6,9-octadecadienoic acid	Δ 6,9-18:2
18:2	linoleic acid	Δ 9,12-18:2 (LA)
18:3 Δ 6,9,12	gamma-linolenic acid	Δ 6,9,12-18:3 (GLA)
18:3 Δ 5,9,12	pinolenic acid	Δ 5,9,12-18:3
18:3	alpha-linolenic acid	Δ 9,12,15-18:3 (ALA)
18:4	stearidonic acid	Δ 6,9,12,15-18:4 (SDA)
20:0	Arachidic acid	
20:1	Eicosenic Acid	
22:0	behehic acid	
22:1	erucic acid	
22:2	Docasadienoic acid	
20:4 ω 6	arachidonic acid	Δ 5,8,11,14-20:4 (ARA)
20:3 ω 6	ω 6-eicosatrienoic dihomo-gamma linolenic	Δ 8,11,14-20:3 (DGLA)
20:5 ω 3	Eicosapentanoic (Timnodonic acid)	Δ 5,8,11,14,17-20:5 (EPA)
20:3 ω 3	ω 3-eicosatrienoic	Δ 11,16,17-20:3
20:4 ω 3	ω 3-eicosatetraenoic	Δ 8,11,14,17-20:4
22:5 ω 3	Docosapentaenoic	Δ 7,10,13,16,19-22:5 (ω 3DPA)
22:6 ω 3	Docosahexaenoic (cervonic acid)	Δ 4,7,10,13,16,19-22:6 (DHA)
24:0	Lignoceric acid	

Taking into account these definitions, the present invention is directed to novel DNA sequences, DNA constructs, methods and compositions are provided which permit modification of the poly-unsaturated long chain fatty acid content of plant cells. Plant cells are transformed with an expression cassette

5 comprising a DNA encoding a polypeptide capable of increasing the amount of one or more PUFA in a plant cell. Desirably, integration constructs may be prepared which provide for integration of the expression cassette into the genome of a host cell. Host cells are manipulated to express a sense or antisense DNA encoding a polypeptide(s) that has desaturase activity. By

10 "desaturase" is intended a polypeptide which can desaturate one or more fatty acids to produce a mono- or poly-unsaturated fatty acid or precursor thereof of interest. By "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification, for example, glycosylation or phosphorylation. The substrate(s) for the expressed enzyme may be produced

15 by the host cell or may be exogenously supplied.

To achieve expression in a host cell, the transformed DNA is operably associated with transcriptional and translational initiation and termination regulatory regions that are functional in the host cell. Constructs comprising the gene to be expressed can provide for integration into the genome of the host cell

20 or can autonomously replicate in the host cell. For production of linoleic acid (LA), the expression cassettes generally used include a cassette which provides for $\Delta 12$ desaturase activity, particularly in a host cell which produces or can take up oleic acid. For production of ALA, the expression cassettes generally used include a cassette which provides for $\Delta 15$ or $\omega 3$ desaturase activity,

25 particularly in a host cell which produces or can take up LA. For production of GLA or SDA, the expression cassettes generally used include a cassette which provides for $\Delta 6$ desaturase activity, particularly in a host cell which produces or can take up LA or ALA, respectively. Production of $\omega 6$ -type unsaturated fatty acids, such as LA or GLA, is favored in a plant capable of producing ALA by

30 inhibiting the activity of a $\Delta 15$ or $\omega 3$ type desaturase; this is accomplished by providing an expression cassette for an antisense $\Delta 15$ or $\omega 3$ transcript, or by

disrupting a $\Delta 15$ or $\omega 3$ desaturase gene. Similarly, production of LA or ALA is favored in a plant having $\Delta 6$ desaturase activity by providing an expression cassette for an antisense $\Delta 6$ transcript, or by disrupting a $\Delta 6$ desaturase gene. Production of oleic acid likewise is favored in a plant having $\Delta 12$ desaturase activity by providing an expression cassette for an antisense $\Delta 12$ transcript, or by disrupting a $\Delta 12$ desaturase gene. For production of ARA, the expression cassette generally used provides for $\Delta 5$ desaturase activity, particularly in a host cell which produces or can take up DGLA. Production of $\omega 6$ -type unsaturated fatty acids, such as ARA, is favored in a plant capable of producing ALA by inhibiting the activity of a $\Delta 15$ or $\omega 3$ type desaturase; this is accomplished by providing an expression cassette for an antisense $\Delta 15$ or $\omega 3$ transcript, or by disrupting a $\Delta 15$ or $\omega 3$ desaturase gene.

TRANSGENIC PLANT PRODUCTION OF FATTY ACIDS

Transgenic plant production of PUFAs offers several advantages over purification from natural sources such as fish or plants. Production of fatty acids from recombinant plants provides the ability to alter the naturally occurring plant fatty acid profile by providing new synthetic pathways in the host or by suppressing undesired pathways, thereby increasing levels of desired PUFAs, or conjugated forms thereof, and decreasing levels of undesired PUFAs. Production of fatty acids in transgenic plants also offers the advantage that expression of desaturase genes in particular tissues and/or plant parts means that greatly increased levels of desired PUFAs in those tissues and/or parts can be achieved, making recovery from those tissues more economical. For example, the desired PUFAs can be expressed in seed; methods of isolating seed oils are well established. In addition to providing a source for purification of desired PUFAs, seed oil components can be manipulated through expression of desaturase genes, either alone or in combination with other genes such as elongases, to provide seed oils having a particular PUFA profile in concentrated form. The concentrated seed oils then can be added to animal milks and/or synthetic or semi-synthetic milks to serve as infant formulas where human

nursing is impossible or undesired, or in cases of malnourishment or disease in both adults and infants.

For production of PUFAs, depending upon the host cell, the availability of substrate, and the desired end product(s), several polypeptides, particularly desaturases, are of interest including those polypeptides which catalyze the conversion of stearic acid to oleic acid, LA to GLA, of ALA to SDA, of oleic acid to LA, or of LA to ALA, which includes enzymes which desaturate at the $\Delta 6$, $\Delta 9$, $\Delta 12$, $\Delta 15$ or $\omega 3$ positions. Considerations for choosing a specific polypeptide having desaturase activity include the pH optimum of the polypeptide, whether the polypeptide is a rate limiting enzyme or a component thereof, whether the desaturase used is essential for synthesis of a desired polyunsaturated fatty acid, and/or co-factors required by the polypeptide. The expressed polypeptide preferably has parameters compatible with the biochemical environment of its location in the host cell. For example, the polypeptide may have to compete for substrate with other enzymes in the host cell. Analyses of the K_m and specific activity of the polypeptide in question therefore are considered in determining the suitability of a given polypeptide for modifying PUFA production in a given host cell. The polypeptide used in a particular situation therefore is one which can function under the conditions present in the intended host cell but otherwise can be any polypeptide having desaturase activity which has the desired characteristic of being capable of modifying the relative production of a desired PUFA. A scheme for the synthesis of arachidonic acid (20:4 $\Delta 5$, 8, 11, 14) from palmitic acid (C_{16}) is shown in Figure 1. A key enzyme in this pathway is a $\Delta 5$ -desaturase which converts DH- γ -linolenic acid (DGLA, eicosatrienoic acid) to ARA. Conversion of α -linolenic acid (ALA) to stearidonic acid by a $\Delta 6$ -desaturase is also shown. Production of PUFAs in addition to ARA, including EPA and DHA is shown in Figure 2. A key enzyme in the synthesis of arachidonic acid (20:4 $\Delta 5$, 8, 11, 14) from stearic acid (C_{18}) is a $\Delta 6$ -desaturase which converts the linoleic acid into γ -linolenic acid. Conversion of α -linolenic acid (ALA) to stearidonic acid by a $\Delta 6$ -desaturase also is shown. For production of ARA, the DNA sequence

used encodes a polypeptide having $\Delta 5$ desaturase activity. In particular instances, this can be coupled with an expression cassette which provides for production of a polypeptide having $\Delta 6$ desaturase activity and, optionally, a transcription cassette providing for production of antisense sequences to a $\Delta 15$ transcription product. The choice of combination of cassettes used depends in part on the PUFA profile of the host cell. Where the host cell $\Delta 5$ -desaturase activity is limiting, overexpression of $\Delta 5$ desaturase alone generally will be sufficient to provide for enhanced ARA production.

SOURCES OF POLYPEPTIDES HAVING DESATURASE ACTIVITY

As sources of polypeptides having desaturase activity and oligonucleotides encoding such polypeptides are organisms which produce a desired poly-unsaturated fatty acid. As an example, microorganisms having an ability to produce ARA can be used as a source of $\Delta 5$ -desaturase genes; microorganisms which GLA or SDA can be used as a source of $\Delta 6$ -desaturase and/or $\Delta 12$ -desaturase genes. Such microorganisms include, for example, those belonging to the genera *Mortierella*, *Conidiobolus*, *Pythium*, *Phytophthora*, *Penicillium*, *Porphyridium*, *Coidosporium*, *Mucor*, *Fusarium*, *Aspergillus*, *Rhodotorula*, and *Entomophthora*. Within the genus *Porphyridium*, of particular interest is *Porphyridium cruentum*. Within the genus *Mortierella*, of particular interest are *Mortierella elongata*, *Mortierella exigua*, *Mortierella hygrophila*, *Mortierella ramanniana*, var. *angulisporea*, and *Mortierella alpina*. Within the genus *Mucor*, of particular interest are *Mucor circinelloides* and *Mucor javanicus*.

DNAs encoding desired desaturases can be identified in a variety of ways. As an example, a source of the desired desaturase, for example genomic or cDNA libraries from *Mortierella*, is screened with detectable enzymatically- or chemically-synthesized probes, which can be made from DNA, RNA, or non-naturally occurring nucleotides, or mixtures thereof. Probes may be enzymatically synthesized from DNAs of known desaturases for normal or

reduced-stringency hybridization methods. Oligonucleotide probes also can be used to screen sources and can be based on sequences of known desaturases, including sequences conserved among known desaturases, or on peptide sequences obtained from the desired purified protein. Oligonucleotide probes
5 based on amino acid sequences can be degenerate to encompass the degeneracy of the genetic code, or can be biased in favor of the preferred codons of the source organism. Oligonucleotides also can be used as primers for PCR from reverse transcribed mRNA from a known or suspected source; the PCR product can be the full length cDNA or can be used to generate a probe to obtain the
10 desired full length cDNA. Alternatively, a desired protein can be entirely sequenced and total synthesis of a DNA encoding that polypeptide performed.

Once the desired genomic or cDNA has been isolated, it can be sequenced by known methods. It is recognized in the art that such methods are subject to errors, such that multiple sequencing of the same region is routine and
15 is still expected to lead to measurable rates of mistakes in the resulting deduced sequence, particularly in regions having repeated domains, extensive secondary structure, or unusual base compositions, such as regions with high GC base content. When discrepancies arise, resequencing can be done and can employ special methods. Special methods can include altering sequencing conditions
20 by using: different temperatures; different enzymes; proteins which alter the ability of oligonucleotides to form higher order structures; altered nucleotides such as ITP or methylated dGTP; different gel compositions, for example adding formamide; different primers or primers located at different distances from the problem region; or different templates such as single stranded DNAs.
25 Sequencing of mRNA can also be employed.

For the most part, some or all of the coding sequence for the polypeptide having desaturase activity is from a natural source. In some situations, however, it is desirable to modify all or a portion of the codons, for example, to enhance expression, by employing host preferred codons. Host preferred
30 codons can be determined from the codons of highest frequency in the proteins expressed in the largest amount in a particular host species of interest. Thus, the

coding sequence for a polypeptide having desaturase activity can be synthesized in whole or in part. All or portions of the DNA also can be synthesized to remove any destabilizing sequences or regions of secondary structure which would be present in the transcribed mRNA. All or portions of the DNA also can be synthesized to alter the base composition to one more preferable in the desired host cell. Methods for synthesizing sequences and bringing sequences together are well established in the literature. *In vitro* mutagenesis and selection, site-directed mutagenesis, or other means can be employed to obtain mutations of naturally occurring desaturase genes to produce a polypeptide having desaturase activity *in vivo* with more desirable physical and kinetic parameters for function in the host cell, such as a longer half-life or a higher rate of production of a desired polyunsaturated fatty acid.

Desirable cDNAs have less than 60% A+T composition, preferably less than 50% A+T composition. On a localized scale of a sliding window of 20 base pairs, it is preferable that there are no localized regions of the cDNA with greater than 75% A+T composition; with a window of 60 base pairs, it is preferable that there are no localized regions of the cDNA with greater than 60%, more preferably no localized regions with greater than 55% A+T composition.

Mortierella alpina Desaturases

Of particular interest are the *Mortierella alpina* $\Delta 5$ -desaturase, $\Delta 6$ -desaturase and $\Delta 12$ -desaturase. The $\Delta 5$ -desaturase has 446 amino acids; the amino acid sequence is shown in Figure 7. The gene encoding the *Mortierella alpina* $\Delta 5$ -desaturase can be expressed in transgenic microorganisms to effect greater synthesis of ARA from DGLA. Other DNAs which are substantially identical in sequence to the *Mortierella alpina* $\Delta 5$ -desaturase DNA, or which encode polypeptides which are substantially identical in sequence to the *Mortierella alpina* $\Delta 5$ -desaturase polypeptide, also can be used. The *Mortierella alpina* $\Delta 6$ -desaturase, has 457 amino acids and a predicted molecular weight of 51.8 kD; the amino acid sequence is shown in Figure 3.

The gene encoding the *Mortierella alpina* $\Delta 6$ -desaturase can be expressed in transgenic plants or animals to effect greater synthesis of GLA from linoleic acid or of stearidonic acid (SDA) from ALA. Other DNAs which are substantially identical in sequence to the *Mortierella alpina* $\Delta 6$ -desaturase DNA, or which encode polypeptides which are substantially identical in sequence to the *Mortierella alpina* $\Delta 6$ -desaturase polypeptide, also can be used.

The *Mortierella alpina* $\Delta 12$ -desaturase has the amino acid sequence shown in Figure 5. The gene encoding the *Mortierella alpina* $\Delta 12$ -desaturase can be expressed in transgenic plants to effect greater synthesis of LA from oleic acid. Other DNAs which are substantially identical to the *Mortierella alpina* $\Delta 12$ -desaturase DNA, or which encode polypeptides which are substantially identical to the *Mortierella alpina* $\Delta 12$ -desaturase polypeptide, also can be used.

By substantially identical in sequence is intended an amino acid sequence or nucleic acid sequence exhibiting in order of increasing preference at least 60%, 80%, 90% or 95% homology to the *Mortierella alpina* $\Delta 5$ -desaturase amino acid sequence or nucleic acid sequence encoding the amino acid sequence. For polypeptides, the length of comparison sequences generally is at least 16 amino acids, preferably at least 20 amino acids, or most preferably 35 amino acids. For nucleic acids, the length of comparison sequences generally is at least 50 nucleotides, preferably at least 60 nucleotides, and more preferably at least 75 nucleotides, and most preferably, 110 nucleotides. Homology typically is measured using sequence analysis software, for example, the Sequence Analysis software package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wisconsin 53705, MEGAlign (DNASar, Inc., 1228 S. Park St., Madison, Wisconsin 53715), and MacVector (Oxford Molecular Group, 2105 S. Bascom Avenue, Suite 200, Campbell, California 95008). Such software matches similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications. Conservative substitutions typically include substitutions within the following groups: glycine and alanine;

valine, isoleucine and leucine; aspartic acid, glutamic acid, asparagine, and glutamine; serine and threonine; lysine and arginine; and phenylalanine and tyrosine. Substitutions may also be made on the basis of conserved hydrophobicity or hydrophilicity (Kyte and Doolittle, *J. Mol. Biol.* 157: 105-132, 1982), or on the basis of the ability to assume similar polypeptide secondary structure (Chou and Fasman, *Adv. Enzymol.* 47: 45-148, 1978).

Other Desaturases

Encompassed by the present invention are related desaturases from the same or other organisms. Such related desaturases include variants of the disclosed $\Delta 5$ -, $\Delta 6$ - and $\Delta 12$ -desaturases that occur naturally within the same or different species of *Mortierella*, as well as homologues of the disclosed $\Delta 5$ -desaturase from other species and evolutionarily related protein having desaturase activity. Also included are desaturases which, although not substantially identical to the *Mortierella alpina* $\Delta 5$ -desaturase, desaturate a fatty acid molecule at carbon 5, 6 or 12, respectively, from the carboxyl end of a fatty acid molecule. Related desaturases can be identified by their ability to function substantially the same as the disclosed desaturases; that is, are still able to effectively convert DGLA to ARA, LA to GLA, ALA to SDA or oleic acid to LA. Related desaturases also can be identified by screening sequence databases for sequences homologous to the disclosed desaturase, by hybridization of a probe based on the disclosed desaturase to a library constructed from the source organism, or by RT-PCR using mRNA from the source organism and primers based on the disclosed desaturase. Such desaturases includes those from humans, *Dictyostelium discoideum* and *Phaeodactylum tricornutum*.

The regions of a desaturase polypeptide important for desaturase activity can be determined through routine mutagenesis, expression of the resulting mutant polypeptides and determination of their activities. Mutants may include deletions, insertions and point mutations, or combinations thereof. A typical functional analysis begins with deletion mutagenesis to determine the N- and C-terminal limits of the protein necessary for function, and then internal deletions,

insertions or point mutants are made to further determine regions necessary for function. Other techniques such as cassette mutagenesis or total synthesis also can be used. Deletion mutagenesis is accomplished, for example, by using exonucleases to sequentially remove the 5' or 3' coding regions. Kits are
5 available for such techniques. After deletion, the coding region is completed by ligating oligonucleotides containing start or stop codons to the deleted coding region after 5' or 3' deletion, respectively. Alternatively, oligonucleotides encoding start or stop codons are inserted into the coding region by a variety of methods including site-directed mutagenesis, mutagenic PCR or by ligation
10 onto DNA digested at existing restriction sites. Internal deletions can similarly be made through a variety of methods including the use of existing restriction sites in the DNA, by use of mutagenic primers via site directed mutagenesis or mutagenic PCR. Insertions are made through methods such as linker-scanning mutagenesis, site-directed mutagenesis or mutagenic PCR. Point mutations are
15 made through techniques such as site-directed mutagenesis or mutagenic PCR.

Chemical mutagenesis can also be used for identifying regions of a desaturase polypeptide important for activity. A mutated construct is expressed, and the ability of the resulting altered protein to function as a desaturase is assayed. Such structure-function analysis can determine which regions may be
20 deleted, which regions tolerate insertions, and which point mutations allow the mutant protein to function in substantially the same way as the native desaturase. All such mutant proteins and nucleotide sequences encoding them are within the scope of the present invention.

EXPRESSION OF DESATURASE GENES

25 Once the DNA encoding a desaturase polypeptide has been obtained, it is placed in a vector capable of replication in a host cell, or is propagated *in vitro* by means of techniques such as PCR or long PCR. Replicating vectors can include plasmids, phage, viruses, cosmids and the like. Desirable vectors include those useful for mutagenesis of the gene of interest or for expression of
30 the gene of interest in host cells. The technique of long PCR has made *in vitro* propagation of large constructs possible, so that modifications to the gene of

interest, such as mutagenesis or addition of expression signals, and propagation of the resulting constructs can occur entirely *in vitro* without the use of a replicating vector or a host cell.

For expression of a desaturase polypeptide, functional transcriptional
5 and translational initiation and termination regions are operably linked to the DNA encoding the desaturase polypeptide. Transcriptional and translational initiation and termination regions are derived from a variety of nonexclusive sources, including the DNA to be expressed, genes known or suspected to be capable of expression in the desired system, expression vectors, chemical
10 synthesis, or from an endogenous locus in a host cell. Expression in a plant tissue and/or plant part presents certain efficiencies, particularly where the tissue or part is one which is easily harvested, such as seed, leaves, fruits, flowers, roots, etc. Expression can be targeted to that location within the plant by using specific regulatory sequences, such as those of USPN 5,463,174,
15 USPN 4,943,674, USPN 5,106,739, USPN 5,175,095, USPN 5,420,034, USPN 5,188,958, and USPN 5,589,379. Alternatively, the expressed protein can be an enzyme which produces a product which may be incorporated, either directly or upon further modifications, into a fluid fraction from the host plant. In the present case, expression of desaturase genes, or antisense desaturase transcripts,
20 can alter the levels of specific PUFAs, or derivatives thereof, found in plant parts and/or plant tissues. The $\Delta 5$ -desaturase polypeptide coding region is expressed either by itself or with other genes, in order to produce tissues and/or plant parts containing higher proportions of desired PUFAs or in which the PUFA composition more closely resembles that of human breast milk (Prieto *et al.*, PCT publication WO 95/24494). The termination region can be derived
25 from the 3' region of the gene from which the initiation region was obtained or from a different gene. A large number of termination regions are known to and have been found to be satisfactory in a variety of hosts from the same and different genera and species. The termination region usually is selected more as
30 a matter of convenience rather than because of any particular property.

The choice of a host cell is influenced in part by the desired PUFA profile of the transgenic cell, and the native profile of the host cell. As an example, for production of linoleic acid from oleic acid, the DNA sequence used encodes a polypeptide having $\Delta 12$ desaturase activity, and for production of GLA from linoleic acid, the DNA sequence used encodes a polypeptide having $\Delta 6$ desaturase activity. Use of a host cell which expresses $\Delta 12$ desaturase activity and lacks or is depleted in $\Delta 15$ desaturase activity, can be used with an expression cassette which provides for overexpression of $\Delta 6$ desaturase alone generally is sufficient to provide for enhanced GLA production in the transgenic cell. Where the host cell expresses $\Delta 9$ desaturase activity, expression of both a $\Delta 12$ - and a $\Delta 6$ -desaturase can provide for enhanced GLA production. In particular instances where expression of $\Delta 6$ desaturase activity is coupled with expression of $\Delta 12$ desaturase activity, it is desirable that the host cell naturally have, or be mutated to have, low $\Delta 15$ desaturase activity. Alternatively, a host cell for $\Delta 6$ desaturase expression may have, or be mutated to have, high $\Delta 12$ desaturase activity.

Expression in a host cell can be accomplished in a transient or stable fashion. Transient expression can occur from introduced constructs which contain expression signals functional in the host cell, but which constructs do not replicate and rarely integrate in the host cell, or where the host cell is not proliferating. Transient expression also can be accomplished by inducing the activity of a regulatable promoter operably linked to the gene of interest, although such inducible systems frequently exhibit a low basal level of expression. Stable expression can be achieved by introduction of a construct that can integrate into the host genome or that autonomously replicates in the host cell. Stable expression of the gene of interest can be selected for through the use of a selectable marker located on or transfected with the expression construct, followed by selection for cells expressing the marker. When stable expression results from integration, integration of constructs can occur randomly within the host genome or can be targeted through the use of constructs containing regions of homology with the host genome sufficient to

target recombination with the host locus. Where constructs are targeted to an endogenous locus, all or some of the transcriptional and translational regulatory regions can be provided by the endogenous locus.

When increased expression of the desaturase polypeptide in the source
5 plant is desired, several methods can be employed. Additional genes encoding the desaturase polypeptide can be introduced into the host organism. Expression from the native desaturase locus also can be increased through homologous recombination, for example by inserting a stronger promoter into the host genome to cause increased expression, by removing destabilizing
10 sequences from either the mRNA or the encoded protein by deleting that information from the host genome, or by adding stabilizing sequences to the mRNA (*see* USPN 4,910,141 and USPN 5,500,365.)

When it is desirable to express more than one different gene, appropriate regulatory regions and expression methods, introduced genes can be propagated
15 in the host cell through use of replicating vectors or by integration into the host genome. Where two or more genes are expressed from separate replicating vectors, it is desirable that each vector has a different means of replication. Each introduced construct, whether integrated or not, should have a different means of selection and should lack homology to the other constructs to maintain
20 stable expression and prevent reassortment of elements among constructs. Judicious choices of regulatory regions, selection means and method of propagation of the introduced construct can be experimentally determined so that all introduced genes are expressed at the necessary levels to provide for synthesis of the desired products.

25 Constructs comprising the gene of interest may be introduced into a host cell by standard techniques. These techniques include transfection, infection, bolistic impact, electroporation, microinjection, scraping, or any other method which introduces the gene of interest into the host cell (*see* USPN 4,743,548, USPN 4,795,855, USPN 5,068,193, USPN 5,188,958, USPN 5,463,174, USPN
30 5,565,346 and USPN 5,565,347). For convenience, a host cell which has been manipulated by any method to take up a DNA sequence or construct will be

referred to as "transformed" or "recombinant" herein. The subject host will have at least have one copy of the expression construct and may have two or more, depending upon whether the gene is integrated into the genome, amplified, or is present on an extrachromosomal element having multiple copy
5 numbers.

The transformed host cell can be identified by selection for a marker contained on the introduced construct. Alternatively, a separate marker construct may be introduced with the desired construct, as many transformation techniques introduce many DNA molecules into host cells. Typically,
10 transformed hosts are selected for their ability to grow on selective media. Selective media may incorporate an antibiotic or lack a factor necessary for growth of the untransformed host, such as a nutrient or growth factor. An introduced marker gene therefor may confer antibiotic resistance, or encode an essential growth factor or enzyme, and permit growth on selective media when
15 expressed in the transformed host cell. Desirably, resistance to kanamycin and the amino glycoside G418 are of interest (*see* USPN 5,034,322). Selection of a transformed host can also occur when the expressed marker protein can be detected, either directly or indirectly. The marker protein may be expressed alone or as a fusion to another protein. The marker protein can be detected by
20 its enzymatic activity; for example β galactosidase can convert the substrate X-gal to a colored product, and luciferase can convert luciferin to a light-emitting product. The marker protein can be detected by its light-producing or modifying characteristics; for example, the green fluorescent protein of *Aequorea victoria* fluoresces when illuminated with blue light. Antibodies can
25 be used to detect the marker protein or a molecular tag on, for example, a protein of interest. Cells expressing the marker protein or tag can be selected, for example, visually, or by techniques such as FACS or panning using antibodies.

The PUFAs produced using the subject methods and compositions may
30 be found in the host plant tissue and/or plant part as free fatty acids or in conjugated forms such as acylglycerols, phospholipids, sulfolipids or

glycolipids, and may be extracted from the host cell through a variety of means well-known in the art. Such means may include extraction with organic solvents, sonication, supercritical fluid extraction using for example carbon dioxide, and physical means such as presses, or combinations thereof. Of particular interest is extraction with hexane or methanol and chloroform. Where desirable, the aqueous layer can be acidified to protonate negatively charged moieties and thereby increase partitioning of desired products into the organic layer. After extraction, the organic solvents can be removed by evaporation under a stream of nitrogen. When isolated in conjugated forms, the products are enzymatically or chemically cleaved to release the free fatty acid or a less complex conjugate of interest, and are then subjected to further manipulations to produce a desired end product. Desirably, conjugated forms of fatty acids are cleaved with potassium hydroxide.

PURIFICATION OF FATTY ACIDS

If further purification is necessary, standard methods can be employed. Such methods include extraction, treatment with urea, fractional crystallization, HPLC, fractional distillation, silica gel chromatography, high speed centrifugation or distillation, or combinations of these techniques. Protection of reactive groups, such as the acid or alkenyl groups, may be done at any step through known techniques, for example alkylation or iodination. Methods used include methylation of the fatty acids to produce methyl esters. Similarly, protecting groups may be removed at any step. Desirably, purification of fractions containing ARA, DHA and EPA is accomplished by treatment with urea and/or fractional distillation.

USES OF FATTY ACIDS

The uses of the fatty acids of subject invention are several. Probes based on the DNAs of the present invention may find use in methods for isolating related molecules or in methods to detect organisms expressing desaturases. When used as probes, the DNAs or oligonucleotides need to be detectable. This is usually accomplished by attaching a label either at an internal site, for

example via incorporation of a modified residue, or at the 5' or 3' terminus. Such labels can be directly detectable, can bind to a secondary molecule that is detectably labeled, or can bind to an unlabelled secondary molecule and a detectably labeled tertiary molecule; this process can be extended as long as is practical to achieve a satisfactorily detectable signal without unacceptable levels of background signal. Secondary, tertiary, or bridging systems can include use of antibodies directed against any other molecule, including labels or other antibodies, or can involve any molecules which bind to each other, for example a biotin-streptavidin/avidin system. Detectable labels typically include radioactive isotopes, molecules which chemically or enzymatically produce or alter light, enzymes which produce detectable reaction products, magnetic molecules, fluorescent molecules or molecules whose fluorescence or light-emitting characteristics change upon binding. Examples of labelling methods can be found in USPN 5,011,770. Alternatively, the binding of target molecules can be directly detected by measuring the change in heat of solution on binding of probe to target via isothermal titration calorimetry, or by coating the probe or target on a surface and detecting the change in scattering of light from the surface produced by binding of target or probe, respectively, as may be done with the BIAcore system.

PUFAs of the subject invention produced by recombinant means find applications in a wide variety of areas. Supplementation of humans or animals with PUFAs in various forms can result in increased levels not only of the added PUFAs, but of their metabolic progeny as well. For example, where the inherent $\Delta 6$ -desaturase pathway is dysfunctional in an individual, treatment with GLA can result not only in increased levels of GLA, but also of downstream products such as ARA and prostaglandins (see Figure 1). Complex regulatory mechanisms can make it desirable to combine various PUFAs, or to add different conjugates of PUFAs, in order to prevent, control or overcome such mechanisms to achieve the desired levels of specific PUFAs in an individual.

PUFAs, or derivatives thereof, made by the disclosed method can be used as dietary supplements, particularly in infant formulas, for patients

undergoing intravenous feeding or for preventing or treating malnutrition. Particular fatty acids such as EPA are used to alter the composition of infant formulas to better replicate the PUFA composition of human breast milk. The predominant triglyceride in human milk has been reported to be 1,3-di-oleoyl-2-palmitoyl, with 2-palmitoyl glycerides reported as better absorbed than 2-oleoyl or 2-lineoyl glycerides (USPN 4,876,107). Typically, human breast milk has a fatty acid profile comprising from about 0.15 % to about 0.36 % as DHA, from about 0.03 % to about 0.13 % as EPA, from about 0.30 % to about 0.88 % as ARA, from about 0.22 % to about 0.67 % as DGLA, and from about 0.27 % to about 1.04 % as GLA. A preferred ratio of GLA:DGLA:ARA in infant formulas is from about 1:1:4 to about 1:1:1, respectively. Amounts of oils providing these ratios of PUFA can be determined without undue experimentation by one of skill in the art. PUFAs, or host cells containing them, also can be used as animal food supplements to alter an animal's tissue or milk fatty acid composition to one more desirable for human or animal consumption.

NUTRITIONAL COMPOSITIONS

The present invention also includes nutritional compositions. Such compositions, for purposes of the present invention, include any food or preparation for human consumption including for enteral or parenteral consumption, which when taken into the body (a) serve to nourish or build up tissues or supply energy and/or (b) maintain, restore or support adequate nutritional status or metabolic function.

The nutritional composition of the present invention comprises at least one oil or acid produced in accordance with the present invention and may either be in a solid or liquid form. Additionally, the composition may include edible macronutrients, vitamins and minerals in amounts desired for a particular use. The amount of such ingredients will vary depending on whether the composition is intended for use with normal, healthy infants, children or adults having specialized needs such as those which accompany certain metabolic conditions (e.g., metabolic disorders).

Examples of macronutrients which may be added to the composition include but are not limited to edible fats, carbohydrates and proteins. Examples of such edible fats include but are not limited to coconut oil, soy oil, and mono- and diglycerides. Examples of such carbohydrates include but are not limited to
5 glucose, edible lactose and hydrolyzed starch. Additionally, examples of proteins which may be utilized in the nutritional composition of the invention include but are not limited to soy proteins, electrodialysed whey , electrodialysed skim milk, milk whey, or the hydrolysates of these proteins.

With respect to vitamins and minerals, the following may be added to
10 the nutritional compositions of the present invention: calcium, phosphorus, potassium, sodium, chloride, magnesium, manganese, iron, copper, zinc, selenium, iodine, and Vitamins A, E, D, C, and the B complex. Other such vitamins and minerals may also be added.

The components utilized in the nutritional compositions of the present
15 invention will of semi-purified or purified origin. By semi-purified or purified is meant a material which has been prepared by purification of a natural material or by synthesis.

Examples of nutritional compositions of the present invention include but are not limited to infant formulas, dietary supplements, and rehydration
20 compositions. Nutritional compositions of particular interest include but are not limited to those utilized for enteral and parenteral supplementation for infants, specialist infant formulae, supplements for the elderly, and supplements for those with gastrointestinal difficulties and/or malabsorption.

Nutritional Compositions

25 A typical nutritional composition of the present invention will contain edible macronutrients, vitamins and minerals in amounts desired for a particular use. The amounts of such ingredients will vary depending on whether the formulation is intended for use with normal, healthy individuals temporarily exposed to stress, or to subjects having specialized needs due to certain chronic
30 or acute disease states (e.g., metabolic disorders). It will be understood by

persons skilled in the art that the components utilized in a nutritional formulation of the present invention are of semi-purified or purified origin. By semi-purified or purified is meant a material that has been prepared by purification of a natural material or by synthesis. These techniques are well known in the art (See, e.g., Code of Federal Regulations for Food Ingredients and Food Processing; Recommended Dietary Allowances, 10th Ed., National Academy Press, Washington, D.C., 1989).

In a preferred embodiment, a nutritional formulation of the present invention is an enteral nutritional product, more preferably an adult or child enteral nutritional product. Accordingly in a further aspect of the invention, a nutritional formulation is provided that is suitable for feeding adults or children who are experiencing stress. The formula comprises, in addition to the PUFAs of the invention; macronutrients, vitamins and minerals in amounts designed to provide the daily nutritional requirements of adults.

The macronutritional components include edible fats, carbohydrates and proteins. Exemplary edible fats are coconut oil, soy oil, and mono- and diglycerides and the PUFA oils of this invention. Exemplary carbohydrates are glucose, edible lactose and hydrolyzed cornstarch. A typical protein source would be soy protein, electrodialysed whey or electrodialysed skim milk or milk whey, or the hydrolysates of these proteins, although other protein sources are also available and may be used. These macronutrients would be added in the form of commonly accepted nutritional compounds in amount equivalent to those present in human milk or an energy basis, i.e., on a per calorie basis.

Methods for formulating liquid and enteral nutritional formulas are well known in the art and are described in detail in the examples.

The enteral formula can be sterilized and subsequently utilized on a ready-to-feed (RTF) basis or stored in a concentrated liquid or a powder. The powder can be prepared by spray drying the enteral formula prepared as indicated above, and the formula can be reconstituted by rehydrating the concentrate. Adult and infant nutritional formulas are well known in the art and commercially available (e.g., Similac®, Ensure®, Jevity® and Alimentum®

from Ross Products Division, Abbott Laboratories). An oil or acid of the present invention can be added to any of these formulas in the amounts described below.

5 The energy density of the nutritional composition when in liquid form, can typically range from about 0.6 Kcal to 3 Kcal per ml. When in solid or powdered form, the nutritional supplement can contain from about 1.2 to more than 9 Kcals per gm, preferably 3 to 7 Kcals per gm. In general, the osmolality of a liquid product should be less than 700 mOsm and more preferably less than 660 mOsm.

10 The nutritional formula would typically include vitamins and minerals, in addition to the PUFAs of the invention, in order to help the individual ingest the minimum daily requirements for these substances. In addition to the PUFAs listed above, it may also be desirable to supplement the nutritional composition with zinc, copper, and folic acid in addition to antioxidants. It is believed that
15 these substances will also provide a boost to the stressed immune system and thus will provide further benefits to the individual. The presence of zinc, copper or folic acid is optional and is not required in order to gain the beneficial effects on immune suppression. Likewise a pharmaceutical composition can be supplemented with these same substances as well.

20 In a more preferred embodiment, the nutritional contains, in addition to the antioxidant system and the PUFA component, a source of carbohydrate wherein at least 5 weight % of said carbohydrate is an indigestible oligosaccharide. In yet a more preferred embodiment, the nutritional composition additionally contains protein, taurine and carnitine.

25 The PUFAs, or derivatives thereof, made by the disclosed method can be used as dietary substitutes, or supplements, particularly infant formulas, for patients undergoing intravenous feeding or for preventing or treating malnutrition. Typically, human breast milk has a fatty acid profile comprising from about 0.15 % to about 0.36 % as DHA, from about 0.03 % to about 0.13 %
30 as EPA, from about 0.30 % to about 0.88 % as ARA, from about 0.22 % to about 0.67 % as DGLA, and from about 0.27 % to about 1.04 % as GLA.

Additionally, the predominant triglyceride in human milk has been reported to be 1,3-di-oleoyl-2-palmitoyl, with 2-palmitoyl glycerides reported as better absorbed than 2-oleoyl or 2-lineoyl glycerides (USPN 4,876,107). Thus, fatty acids such as ARA, DGLA, GLA and/or EPA produced by the invention can be used to alter the composition of infant formulas to better replicate the PUFA composition of human breast milk. In particular, an oil composition for use in a pharmacologic or food supplement, particularly a breast milk substitute or supplement, will preferably comprise one or more of ARA, DGLA and GLA. More preferably the oil will comprise from about 0.3 to 30% ARA, from about 0.2 to 30% DGLA, and from about 0.2 to about 30% GLA.

In addition to the concentration, the ratios of ARA, DGLA and GLA can be adapted for a particular given end use. When formulated as a breast milk supplement or substitute, an oil composition which contains two or more of ARA, DGLA and GLA will be provided in a ratio of about 1:19:30 to about 6:1:0.2, respectively. For example, the breast milk of animals can vary in ratios of ARA:DGLA:DGL ranging from 1:19:30 to 6:1:0.2, which includes intermediate ratios which are preferably about 1:1:1, 1:2:1, 1:1:4. When produced together in a host cell, adjusting the rate and percent of conversion of a precursor substrate such as GLA and DGLA to ARA can be used to precisely control the PUFA ratios. For example, a 5% to 10% conversion rate of DGLA to ARA can be used to produce an ARA to DGLA ratio of about 1:19, whereas a conversion rate of about 75% to 80% can be used to produce an ARA to DGLA ratio of about 6:1. Therefore, whether in a cell culture system or in a host animal, regulating the timing, extent and specificity of desaturase expression as described can be used to modulate the PUFA levels and ratios. Depending on the expression system used, e.g., cell culture or an animal expressing oil(s) in its milk, the oils also can be isolated and recombined in the desired concentrations and ratios. Amounts of oils providing these ratios of PUFA can be determined following standard protocols. PUFAs, or host cells containing them, also can be used as animal food supplements to alter an animal's tissue or milk fatty acid composition to one more desirable for human or animal consumption.

For dietary supplementation, the purified PUFAs, or derivatives thereof, may be incorporated into cooking oils, fats or margarines formulated so that in normal use the recipient would receive the desired amount. The PUFAs may also be incorporated into infant formulas, nutritional supplements or other food products, and may find use as anti-inflammatory or cholesterol lowering agents.

Pharmaceutical Compositions

The present invention also encompasses a pharmaceutical composition comprising one or more of the acids and/or resulting oils produced in accordance with the methods described herein. More specifically, such a pharmaceutical composition may comprise one or more of the acids and/or oils as well as a standard, well-known, non-toxic pharmaceutically acceptable carrier, adjuvant or vehicle such as, for example, phosphate buffered saline, water, ethanol, polyols, vegetable oils, a wetting agent or an emulsion such as a water/oil emulsion. The composition may be in either a liquid or solid form.

For example, the composition may be in the form of a tablet, capsule, ingestible liquid or powder, injectible, or topical ointment or cream.

Possible routes of administration include, for example, oral, rectal and parenteral. The route of administration will, of course, depend upon the desired effect. For example, if the composition is being utilized to treat rough, dry, or aging skin, to treat injured or burned skin, or to treat skin or hair affected by a disease or condition, it may perhaps be applied topically.

The dosage of the composition to be administered to the patient may be determined by one of ordinary skill in the art and depends upon various factors such as weight of the patient, age of the patient, immune status of the patient, etc.

With respect to form, the composition may be, for example, a solution, a dispersion, a suspension, an emulsion or a sterile powder which is then reconstituted.

Additionally, the composition of the present invention may be utilized for cosmetic purposes. It may be added to pre-existing cosmetic compositions such that a mixture is formed or may be used as a sole composition.

Pharmaceutical compositions may be utilized to administer the PUFA
5 component to an individual. Suitable pharmaceutical compositions may comprise physiologically acceptable sterile aqueous or non-aqueous solutions, dispersions, suspensions or emulsions and sterile powders for reconstitution into sterile solutions or dispersions for ingestion. Examples of suitable aqueous and non-aqueous carriers, diluents, solvents or vehicles include water, ethanol,
10 polyols (propyleneglycol, polyethyleneglycol, glycerol, and the like), suitable mixtures thereof, vegetable oils (such as olive oil) and injectable organic esters such as ethyl oleate. Proper fluidity can be maintained, for example, by the maintenance of the required particle size in the case of dispersions and by the use of surfactants. It may also be desirable to include isotonic agents, for
15 example sugars, sodium chloride and the like. Besides such inert diluents, the composition can also include adjuvants, such as wetting agents, emulsifying and suspending agents, sweetening, flavoring and perfuming agents.

Suspensions, in addition to the active compounds, may contain suspending agents, as for example, ethoxylated isostearyl alcohols,
20 polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth or mixtures of these substances, and the like.

Solid dosage forms such as tablets and capsules can be prepared using techniques well known in the art. For example, PUFAs of the invention can be
25 tableted with conventional tablet bases such as lactose, sucrose, and cornstarch in combination with binders such as acacia, cornstarch or gelatin, disintegrating agents such as potato starch or alginic acid and a lubricant such as stearic acid or magnesium stearate. Capsules can be prepared by incorporating these excipients into a gelatin capsule along with the antioxidants and the PUFA
30 component. The amount of the antioxidants and PUFA component that should

be incorporated into the pharmaceutical formulation should fit within the guidelines discussed above.

As used in this application, the term "treat" refers to either preventing, or reducing the incidence of, the undesired occurrence. For example, to treat
5 immune suppression refers to either preventing the occurrence of this suppression or reducing the amount of such suppression. The terms "patient" and "individual" are being used interchangeably and both refer to an animal. The term "animal" as used in this application refers to any warm-blooded mammal including, but not limited to, dogs, humans, monkeys, and apes. As
10 used in the application the term "about" refers to an amount varying from the stated range or number by a reasonable amount depending upon the context of use. Any numerical number or range specified in the specification should be considered to be modified by the term about.

"Dose" and "serving" are used interchangeably and refer to the amount
15 of the nutritional or pharmaceutical composition ingested by the patient in a single setting and designed to deliver effective amounts of the antioxidants and the structured triglyceride. As will be readily apparent to those skilled in the art, a single dose or serving of the liquid nutritional powder should supply the amount of antioxidants and PUFAs discussed above. The amount of the dose or
20 serving should be a volume that a typical adult can consume in one sitting. This amount can vary widely depending upon the age, weight, sex or medical condition of the patient. However as a general guideline, a single serving or dose of a liquid nutritional produce should be considered as encompassing a volume from 100 to 600 ml, more preferably from 125 to 500 ml and most
25 preferably from 125 to 300 ml.

The PUFAs of the present invention may also be added to food even when supplementation of the diet is not required. For example, the composition may be added to food of any type including but not limited to margarines, modified butters, cheeses, milk, yogurt, chocolate, candy, snacks, salad oils,
30 cooking oils, cooking fats, meats, fish and beverages.

Pharmaceutical Applications

For pharmaceutical use (human or veterinary), the compositions are generally administered orally but can be administered by any route by which they may be successfully absorbed, e.g., parenterally (i.e. subcutaneously, intramuscularly or intravenously), rectally or vaginally or topically, for example, as a skin ointment or lotion. The PUFAs of the present invention may be administered alone or in combination with a pharmaceutically acceptable carrier or excipient. Where available, gelatin capsules are the preferred form of oral administration. Dietary supplementation as set forth above also can provide an oral route of administration. The unsaturated acids of the present invention may be administered in conjugated forms, or as salts, esters, amides or prodrugs of the fatty acids. Any pharmaceutically acceptable salt is encompassed by the present invention; especially preferred are the sodium, potassium or lithium salts. Also encompassed are the N-alkylpolyhydroxamine salts, such as N-methyl glucamine, found in PCT publication WO 96/33155. The preferred esters are the ethyl esters. As solid salts, the PUFAs also can be administered in tablet form. For intravenous administration, the PUFAs or derivatives thereof may be incorporated into commercial formulations such as Intralipids. The typical normal adult plasma fatty acid profile comprises 6.64 to 9.46% of ARA, 1.45 to 3.11% of DGLA, and 0.02 to 0.08% of GLA. These PUFAs or their metabolic precursors can be administered, either alone or in mixtures with other PUFAs, to achieve a normal fatty acid profile in a patient. Where desired, the individual components of formulations may be individually provided in kit form, for single or multiple use. A typical dosage of a particular fatty acid is from 0.1 mg to 20 g, or even 100 g daily, and is preferably from 10 mg to 1, 2, 5 or 10 g daily as required, or molar equivalent amounts of derivative forms thereof. Parenteral nutrition compositions comprising from about 2 to about 30 weight percent fatty acids calculated as triglycerides are encompassed by the present invention; preferred is a composition having from about 1 to about 25 weight percent of the total PUFA composition as GLA (USPN 5,196,198). Other vitamins, and particularly fat-soluble vitamins such as vitamin A, D, E and L-carnitine can optionally be included. Where desired, a

preservative such as α tocopherol may be added, typically at about 0.1% by weight.

Suitable pharmaceutical compositions may comprise physiologically acceptable sterile aqueous or non-aqueous solutions, dispersions, suspensions or emulsions and sterile powders for reconstitution into sterile injectible solutions or dispersions. Examples of suitable aqueous and non-aqueous carriers, diluents, solvents or vehicles include water, ethanol, polyols (propyleneglyol, polyethyleneglycol, glycerol, and the like), suitable mixtures thereof, vegetable oils (such as olive oil) and injectable organic esters such as ethyl oleate. Proper fluidity can be maintained, for example, by the maintenance of the required particle size in the case of dispersions and by the use of surfactants. It may also be desirable to include isotonic agents, for example sugars, sodium chloride and the like. Besides such inert diluents, the composition can also include adjuvants, such as wetting agents, emulsifying and suspending agents, sweetening, flavoring and perfuming agents.

Suspensions in addition to the active compounds, may contain suspending agents, as for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, or mixtures of these substances and the like.

An especially preferred pharmaceutical composition contains diacetyltartaric acid esters of mono- and diglycerides dissolved in an aqueous medium or solvent. Diacetyltartaric acid esters of mono- and diglycerides have an HLB value of about 9-12 and are significantly more hydrophilic than existing antimicrobial lipids that have HLB values of 2-4. Those existing hydrophobic lipids cannot be formulated into aqueous compositions. As disclosed herein, those lipids can now be solubilized into aqueous media in combination with diacetyltartaric acid esters of mono- and diglycerides. In accordance with this embodiment, diacetyltartaric acid esters of mono- and diglycerides (e.g., DATEM-C12:0) is melted with other active antimicrobial lipids (e.g., 18:2 and 12:0 monoglycerides) and mixed to obtain a homogeneous mixture.

Homogeneity allows for increased antimicrobial activity. The mixture can be completely dispersed in water. This is not possible without the addition of diacetyltartaric acid esters of mono- and diglycerides and premixing with other monoglycerides prior to introduction into water. The aqueous composition can
5 then be admixed under sterile conditions with physiologically acceptable diluents, preservatives, buffers or propellants as may be required to form a spray or inhalant.

The present invention also encompasses the treatment of numerous disorders with fatty acids. Supplementation with PUFAs of the present
10 invention can be used to treat restenosis after angioplasty. Symptoms of inflammation, rheumatoid arthritis, and asthma and psoriasis can be treated with the PUFAs of the present invention. Evidence indicates that PUFAs may be involved in calcium metabolism, suggesting that PUFAs of the present invention may be used in the treatment or prevention of osteoporosis and of
15 kidney or urinary tract stones.

The PUFAs of the present invention can be used in the treatment of cancer. Malignant cells have been shown to have altered fatty acid compositions; addition of fatty acids has been shown to slow their growth and cause cell death, and to increase their susceptibility to chemotherapeutic agents.
20 GLA has been shown to cause reexpression on cancer cells of the E-cadherin cellular adhesion molecules, loss of which is associated with aggressive metastasis. Clinical testing of intravenous administration of the water soluble lithium salt of GLA to pancreatic cancer patients produced statistically significant increases in their survival. PUFA supplementation may also be
25 useful for treating cachexia associated with cancer.

The PUFAs of the present invention can also be used to treat diabetes (USPN 4,826,877; Horrobin *et al.*, Am. J. Clin. Nutr. Vol. 57 (Suppl.), 732S-737S). Altered fatty acid metabolism and composition has been demonstrated in diabetic animals. These alterations have been suggested to be involved in
30 some of the long-term complications resulting from diabetes, including retinopathy, neuropathy, nephropathy and reproductive system damage.

Primrose oil, which contains GLA, has been shown to prevent and reverse diabetic nerve damage.

The PUFAs of the present invention can be used to treat eczema, reduce blood pressure and improve math scores. Essential fatty acid deficiency has
5 been suggested as being involved in eczema, and studies have shown beneficial effects on eczema from treatment with GLA. GLA has also been shown to reduce increases in blood pressure associated with stress, and to improve performance on arithmetic tests. GLA and DGLA have been shown to inhibit platelet aggregation, cause vasodilation, lower cholesterol levels and inhibit
10 proliferation of vessel wall smooth muscle and fibrous tissue (Brenner *et al.*, Adv. Exp. Med. Biol. Vol. 83, p. 85-101, 1976). Administration of GLA or DGLA, alone or in combination with EPA, has been shown to reduce or prevent gastro-intestinal bleeding and other side effects caused by non-steroidal anti-inflammatory drugs (USPN 4,666,701). GLA and DGLA have also been shown
15 to prevent or treat endometriosis and premenstrual syndrome (USPN 4,758,592) and to treat myalgic encephalomyelitis and chronic fatigue after viral infections (USPN 5,116,871).

Further uses of the PUFAs of this invention include use in treatment of AIDS, multiple sclerosis, acute respiratory syndrome, hypertension and
20 inflammatory skin disorders. The PUFAs of the inventions also can be used for formulas for general health as well as for geriatric treatments.

Veterinary Applications

It should be noted that the above-described pharmaceutical and nutritional compositions may be utilized in connection with animals, as well as
25 humans, as animals experience many of the same needs and conditions as human. For example, the oil or acids of the present invention may be utilized in animal feed supplements or as animal feed substitutes.

The following examples are presented by way of illustration, not of limitation.

Examples

- Example 1 Isolation of $\Delta 5$ Desaturase Nucleotide Sequence from *Mortierella alpina*
- 5 Example 2 Isolation of $\Delta 6$ Desaturase Nucleotide Sequence from *Mortierella alpina*
- Example 3 Identification of $\Delta 6$ Desaturases Homologues to the *Mortierella alpina* Δ Desaturase
- Example 4 Isolation of D-12 Desaturase Nucleotide Sequence from *Mortierella alpina*
- 10 Example 5 Isolation of Cytochrome b5 Reductase Nucleotide Sequence from *Mortierella alpina*
- Example 6 Expression of *M. alpina* Desaturase Clones in Baker's Yeast
- Example 7 Fatty Acid Analysis of Leaves from Ma29 Transgenic *Brassica* Plants
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- Example 9 Expression of *M. alpina* $\Delta 12$ desaturase in *Brassica napus*
- 20 Example 10 Simultaneous expression of *M. alpina* $\Delta 6$ and $\Delta 12$ desaturases in *Brassica napus*
- Example 11 Simultaneous expression of *M. alpina* $\Delta 5$ and $\Delta 6$ desaturases in *Brassica napus*
- 25 Example 12 Simultaneous expression of *M. alpina* $\Delta 5$, $\Delta 6$ and $\Delta 12$ desaturases in *Brassica napus*
- Example 13 Stereospecific Distribution of $\Delta 6$ -Desaturated Oils
- Example 14 Fatty Acid Compositions of Transgenic Plants

Example 15 Combined Expression of $\Delta 6$ and $\Delta 12$ Desaturases in *B. napus* Achieved by Crossing

Example 16 Expression of *M. alpina* desaturases in soybean

Example 17 Human Desaturase Gene Sequences

5

Example 1

Isolation of a $\Delta 5$ -desaturase Nucleotide Sequence from *Mortierella alpina*

Mortierella alpina produces arachidonic acid (ARA, 20:4) from the precursor 20:3 by a $\Delta 5$ -desaturase. A nucleotide sequence encoding the $\Delta 5$ -desaturase from *Mortierella alpina* (see Figure 7) was obtained through PCR
10 amplification using *M. alpina* 1st strand cDNA and degenerate oligonucleotide primers corresponding to amino acid sequences conserved between $\Delta 6$ -desaturases from *Synechocystis* and *Spirulina*. The procedure used was as follows:

Total RNA was isolated from a 3 day old PUFA-producing culture of
15 *Mortierella alpina* using the protocol of Hoge *et al.* (1982) *Experimental Mycology* 6:225-232. The RNA was used to prepare double-stranded cDNA using BRL's lambda-ZipLox system, following the manufacturer's instructions. Several size fractions of the *M. alpina* cDNA were packaged separately to yield libraries with different average-sized inserts. The "full-length" library contains
20 approximately 3×10^6 clones with an average insert size of 1.77 kb. The "sequencing-grade" library contains approximately 6×10^5 clones with an average insert size of 1.1 kb.

5 μ g of total RNA was reverse transcribed using BRL Superscript RTase and the primer TSyn 5'-CAAGCTTCTGCAGGAGCTCTTTTTTTTTTTTTTTT-
25 3' (SEQ ID NO:19.) Degenerate oligonucleotides were designed to regions conserved between the two cyanobacterial $\Delta 6$ -desaturase sequences. The specific primers used were:

D6DESAT-F3 (SEQ ID NO:20)

5'-CUACUACUACUACAYCAYACOTAYACOAAYAT-3'

D6DESAT-R3 (SEQ ID NO:21)

5'-CAUCAUCAUCAUOGGRAAOARRTGRTG-3'

5 where Y=C+T, R=A+G, and O=I+C. PCR amplification was carried out in a 25µl volume containing: template derived from 40 ng total RNA, 2 pM each primer, 200 µM each deoxyribonucleotide triphosphate, 60 mM Tris-Cl, pH 8.5, 15 mM (NH₄)₂SO₄, 2 mM MgCl₂. Samples were subjected to an initial desaturation step of 95 degrees (all temperatures Celsius) for 5 minutes, then
10 held at 72 degrees while 0.2 U of Taq polymerase were added. PCR thermocycling conditions were as follows: 94 degrees for 1 min., 45 degrees for 1.5 min., 72 degrees for 2 min. PCR was continued for 35 cycles. PCR using these primers on the *M. alpina* first-strand cDNA produced a 550 bp reaction product. Comparison of the deduced amino acid sequence of the *M.*
15 *alpina* PCR fragment revealed regions of homology with Δ6-desaturases (see Figure 4). However, there was only about 28% identity over the region compared. The deduced amino acid sequence is presented in SEQ ID NO:14.

The PCR product was used as a probe to isolate corresponding cDNA clones from a *M. alpina* library. The longest cDNA clone, Ma29, was
20 designated pCGN5521 and has been completely sequenced on both strands. The cDNA is contained as a 1481 bp insert in the vector pZL1 (Bethesda Research Laboratories) and, beginning with the first ATG, contains an open reading frame encoding 446 amino acids. The reading frame contains the sequence deduced from the PCR fragment. The sequence of the cDNA insert
25 was found to contain regions of homology to Δ6-desaturases (see Figure 8). For example, three conserved "histidine boxes" (that have been observed in other membrane-bound desaturases (Okuley *et al.*, (1994) *The Plant Cell* 6:147-158)) were found to be present in the *Mortierella* sequence at amino acid positions 171-175, 207-212, and 387-391 (see Figure 5A-5D). However, the typical
30 "HXXHH" amino acid motif for the third histidine box for the *Mortierella*

desaturase was found to be QXXHH. The amino-terminus of the encoded protein, showed significant homology to cytochrome b5 proteins. Thus, the *Mortierella* cDNA clone appears to represent a fusion between a cytochrome b5 and a fatty acid desaturase. Since cytochrome b5 is believed to function as the electron donor for membrane-bound desaturase enzymes, it is possible that the N-terminal cytochrome b5 domain of this desaturase protein is involved in its function. This may be advantageous when expressing the desaturase in heterologous systems for PUFA production.

Example 2

10 Isolation of $\Delta 6$ Desaturase Nucleotide Sequence from *Mortierella alpina*

A nucleic acid sequence from a partial cDNA clone, Ma524, encoding a $\Delta 6$ fatty acid desaturase from *Mortierella alpina* was obtained by random sequencing of clones from the *M. alpina* cDNA library described in Example 1. cDNA-containing plasmids were excised as follows:

15 Five μ l of phage were combined with 100 μ l of *E. coli* DH10B(ZIP) grown in ECLB plus 10 μ g/ml kanamycin, 0.2% maltose, and 10 mM $MgSO_4$ and incubated at 37 degrees for 15 minutes. 0.9 ml SOC was added and 100 μ l of the bacteria immediately plated on each of 10 ECLB + 50 μ g Pen plates. No 45 minute recovery time was needed. The plates were incubated overnight at 37
20 degrees. Colonies were picked into ECLB + 50 μ g Pen media for overnight cultures to be used for making glycerol stocks and miniprep DNA. An aliquot of the culture used for the miniprep is stored as a glycerol stock. Plating on ECLB + 50 μ g Pen/ml resulted in more colonies and a greater proportion of colonies containing inserts than plating on 100 μ g/ml Pen.

25 Random colonies were picked and plasmid DNA purified using Qiagen miniprep kits. DNA sequence was obtained from the 5' end of the cDNA insert and compared to the databases using the BLAST algorithm. Ma524 was identified as a putative $\Delta 6$ desaturase based on DNA sequence homology to previously identified $\Delta 6$ desaturases. A full-length cDNA clone was isolated

from the *M. alpina* library. The abundance of this clone appears to be slightly (2X) less than Ma29. Ma524 displays significant homology to a portion of a *Caenorhabditis elegans* cosmid, WO6D2.4, a cytochrome b5/desaturase fusion protein from sunflower, and the two $\Delta 6$ desaturases in the public databanks
5 those from *Synechocystis* and *Spirulina*.

In addition, Ma524 shows significant homology to the borage $\Delta 6$ -desaturase sequence (PCT publication WO 96/21022). Ma524 thus appears to encode a $\Delta 6$ -desaturase that is related to the borage and algal $\Delta 6$ -desaturases. It should be noted that, although the amino acid sequences of Ma524 and the
10 borage $\Delta 6$ are similar, the base composition of the cDNAs is quite different: the borage cDNA has an overall base composition of 60 % A+T, with some regions exceeding 70 %, while Ma524 has an average of 44 % A+T base composition, with no regions exceeding 60 %. This may have implications for expressing the cDNAs in microorganisms or animals which favor different base compositions.
15 It is known that poor expression of recombinant genes can occur when the host has a very different base composition from that of the introduced gene. Speculated mechanisms for such poor expression include decreased stability or translatability of the mRNA.

Example 3

20 Identification of $\Delta 6$ -desaturases Homologous to the *Mortierella alpina* $\Delta 6$ -desaturase

Nucleic acid sequences that encode putative $\Delta 6$ -desaturases were identified through a BLASTX search of the est databases through NCBI using the Ma524 amino acid sequence. Several sequences showed significant
25 homology. In particular, the deduced amino acid sequence of two *Arabidopsis thaliana* sequences, (accession numbers F13728 and T42806) showed homology to two different regions of the deduced amino acid sequence of Ma524. The following PCR primers were designed: ATTS4723-FOR (complementary to F13728) 5'-CUACUACUACUAGGAGTCCTCTA
30 CGGTGTTTTG, SEQ ID NO:22, and T42806-REV (complementary to

T42806) 5' CAUCAUCAUATGATGCTCAAGCTGAACTG, SEQ ID NO:23. Five μ g of total RNA isolated from developing siliques of *Arabidopsis thaliana* was reverse transcribed using BRL Superscript RTase and the primer TSyn 5'-CCAAGCTTCTGCAGGAGCTCTTTTTTTTTTTTTTTT-3', (SEQ ID NO:24). PCR was carried out in a 50 μ l volume containing: template derived from 25 ng total RNA, 2 pM each primer, 200 μ M each deoxyribonucleotide triphosphate, 60 mM Tris-Cl, pH 8.5, 15 mM $(\text{NH}_4)_2\text{SO}_4$, 2 mM MgCl_2 , 0.2 U Taq Polymerase. Cycle conditions were as follows: 94 degrees for 30 sec., 50 degrees for 30 sec., 72 degrees for 30 sec. PCR was continued for 35 cycles followed by an additional extension at 72 degrees for 7 minutes. PCR resulted in a fragment of ~750 base pairs which was subsequently subcloned, named 12-5, and sequenced. Each end of this fragment corresponds to the *Arabidopsis* est from which the PCR primers were derived. This is the sequence named 12-5. The deduced amino acid sequence of 12-5 is compared to that of Ma524 and ests from human (W28140), mouse (W53753), and *C. elegans* (R05219) in Figure 4. Based on homology, these sequences represent desaturase polypeptides. The full-length genes can be cloned using probes based on the est sequences. The genes can then be placed in expression vectors and expressed in host cells and their specific $\Delta 6$ - or other desaturase activity can be determined as described below.

Example 4

Isolation of Δ -12 Desaturase Nucleotide Sequence from *Mortierella alpina*

Based on the fatty acids it accumulates, *Mortierella alpina* has an $\omega 6$ type desaturase. The $\omega 6$ desaturase is responsible for the production of linoleic acid (18:2) from oleic acid (18:1). Linoleic acid (18:2) is a substrate for a $\Delta 6$ desaturase. This experiment was designed to determine if *Mortierella alpina* has a $\Delta 12$ -desaturase polypeptide, and if so, to identify the corresponding nucleotide sequence. A random colony from the *M. alpina* sequencing grade library, Ma648, was sequenced and identified as a putative desaturase based on DNA sequence homology to previously identified desaturases, as described for

Ma524 (*see* Example 2). The deduced amino acid sequence from the 5' end of the Ma648 cDNA displays significant homology to soybean microsomal ω 6 (Δ 12) desaturase (accession #L43921) as well as castor bean oleate 12-hydroxylase (accession #U22378). In addition, homology is observed to a
5 variety of other ω 6 (Δ 12) and ω 3 (Δ 15) fatty acid desaturase sequences.

Example 5

Isolation of Cytochrome b5 Reductase Nucleotide Sequence from *Mortierella alpina*

A nucleic acid sequence encoding a cytochrome b5 reductase from
10 *Mortierella alpina* was obtained as follows. A cDNA library was constructed based on total RNA isolated from *Mortierella alpina* as described in Example 1. DNA sequence was obtained from the 5' and 3' ends of one of the clones, M12-27. A search of public databanks with the deduced amino acid sequence of the 3' end of M12-27 (*see* Figure 5) revealed significant homology to known
15 cytochrome b5 reductase sequences. Specifically, over a 49 amino acid region, the *Mortierella* clone shares 55% identity (73% homology) with a cytochrome b5 reductase from pig (*see* Figure 4).

Example 6

Expression of *M. alpina* Desaturase Clones in Baker's Yeast Yeast Transformation

Lithium acetate transformation of yeast was performed according to standard protocols (*Methods in Enzymology*, Vol. 194, p. 186-187, 1991). Briefly, yeast were grown in YPD at 30°C. Cells were spun down, resuspended in TE, spun down again, resuspended in TE containing 100 mM lithium acetate,
25 spun down again, and resuspended in TE/lithium acetate. The resuspended yeast were incubated at 30°C for 60 minutes with shaking. Carrier DNA was added, and the yeast were aliquoted into tubes. Transforming DNA was added, and the tubes were incubated for 30 min. at 30°C. PEG solution (35% (w/v) PEG 4000, 100 mM lithium acetate, TE pH7.5) was added followed by a 50

min. incubation at 30°C. A 5 min. heat shock at 42°C was performed, the cells were pelleted, washed with TE, pelleted again and resuspended in TE. The resuspended cells were then plated on selective media.

Desaturase Expression in Transformed Yeast

5 cDNA clones from *Mortierella alpina* were screened for desaturase activity in baker's yeast. A canola $\Delta 15$ -desaturase (obtained by PCR using 1st strand cDNA from *Brassica napus* cultivar 212/86 seeds using primers based on the published sequence (Arondel *et al. Science* 258:1353-1355)) was used as a positive control. The $\Delta 15$ -desaturase gene and the gene from cDNA clone
10 Ma29 was put in the expression vector pYES2 (Invitrogen), resulting in plasmids pCGR-2 and pCGR-4, respectively. These plasmids were transfected into *S. cerevisiae* yeast strain 334 and expressed after induction with galactose and in the presence of substrates that allowed detection of specific desaturase activity. The control strain was *S. cerevisiae* strain 334 containing the unaltered
15 pYES2 vector. The substrates used, the products produced and the indicated desaturase activity were: DGLA (conversion to ARA would indicate $\Delta 5$ -desaturase activity), linoleic acid (conversion to GLA would indicate $\Delta 6$ -desaturase activity; conversion to ALA would indicate $\Delta 15$ -desaturase activity), oleic acid (an endogenous substrate made by *S. cerevisiae*, conversion to
20 linoleic acid would indicate $\Delta 12$ -desaturase activity, which *S. cerevisiae* lacks), or ARA (conversion to EPA would indicate $\Delta 17$ -desaturase activity). The results are provided in Table 1 below. The lipid fractions were extracted as follows: Cultures were grown for 48-52 hours at 15°C. Cells were pelleted by centrifugation, washed once with sterile ddH₂O, and repelleted. Pellets were
25 vortexed with methanol; chloroform was added along with tritridecanoin (as an internal standard). The mixtures were incubated for at least one hour at room temperature or at 4°C overnight. The chloroform layer was extracted and filtered through a Whatman filter with one gram of anhydrous sodium sulfate to remove particulates and residual water. The organic solvents were evaporated
30 at 40°C under a stream of nitrogen. The extracted lipids were then derivatized to fatty acid methyl esters (FAME) for gas chromatography analysis (GC) by

adding 2 ml of 0.5 N potassium hydroxide in methanol to a closed tube. The samples were heated to 95°C to 100°C for 30 minutes and cooled to room temperature. Approximately 2 ml of 14 % boron trifluoride in methanol was added and the heating repeated. After the extracted lipid mixture cooled, 2 ml
5 of water and 1 ml of hexane were added to extract the FAME for analysis by GC. The percent conversion was calculated by dividing the product produced by the sum of (the product produced and the substrate added) and then multiplying by 100. To calculate the oleic acid percent conversion, as no
10 substrate was added, the total linoleic acid produced was divided by the sum of (oleic acid and linoleic acid produced), then multiplying by 100.

Table 1***M. alpina* Desaturase Expression in Baker's Yeast**

CLONE	TYPE OF ENZYME ACTIVITY	% CONVERSION OF SUBSTRATE
pCGR-2	$\Delta 6$	0 (18:2 to 18:3 ω 6)
(canola $\Delta 15$ desaturase)	$\Delta 15$	16.3 (18:2 to 18:3 ω 3)
	$\Delta 5$	2.0 (20:3 to 20:4 ω 6)
	$\Delta 17$	2.8 (20:4 to 20:5 ω 3)
	$\Delta 12$	1.8 (18:1 to 18:2 ω 6)
pCGR-4	$\Delta 6$	0
(M. alpina $\Delta 6$ -like, Ma29)	$\Delta 15$	0
	$\Delta 5$	15.3
	$\Delta 17$	0.3
	$\Delta 12$	3.3
pCGR-7	$\Delta 6$	0
(M. alpina $\Delta 12$ -like, Ma648)	$\Delta 15$	3.8
	$\Delta 5$	2.2
	$\Delta 17$	0
	$\Delta 12$	63.4

The $\Delta 15$ -desaturase control clone exhibited 16.3% conversion of the substrate. The pCGR-4 clone expressing the Ma29 cDNA converted 15.3% of the 20:3 substrate to 20:4 ω 6, indicating that the gene encodes a $\Delta 5$ -desaturase. The background (non-specific conversion of substrate) was between 0-3% in these cases. The pCGR-5 clone expressing the Ma524 cDNA showed 6% conversion of the substrate to GLA, indicating that the gene encodes a $\Delta 6$ -desaturase. The pCGR-7 clone expressing the Ma648 cDNA converted 63.4% conversion of the substrate to LA, indicating that the gene encodes a $\Delta 12$ -desaturase. Substrate inhibition of activity was observed by using different concentrations of the substrate. When substrate was added to 100 μ M, the percent conversion to product dropped as compared to when substrate was added to 25 μ M (see below). These data show that desaturases with different

substrate specificities can be expressed in a heterologous system and used to produce PUFAs.

Table 2 represents fatty acids of interest as a percent of the total lipid extracted from the yeast host *S. cerevisiae* 334 with the indicated plasmid. No
5 glucose was present in the growth media. Affinity gas chromatography was used to separate the respective lipids. GC/MS was employed to verify the identity of the product(s). The expected product for the *B. napus* $\Delta 15$ -desaturase, α -linolenic acid, was detected when its substrate, linoleic acid, was added exogenously to the induced yeast culture. This finding demonstrates that
10 yeast expression of a desaturase gene can produce functional enzyme and detectable amounts of product under the current growth conditions. Both exogenously added substrates were taken up by yeast, although slightly less of the longer chain PUFA, dihomogamma-linolenic acid (20:3), was incorporated into yeast than linoleic acid (18:2) when either was added in free form to the induced
15 yeast cultures. gamma-linolenic acid was detected when linoleic acid was present during induction and expression of *S. cerevisiae* 334 (pCGR-5). The presence of this PUFA demonstrates $\Delta 6$ -desaturase activity from pCGR-5 (MA524). Linoleic acid, identified in the extracted lipids from expression of *S. cerevisiae* 334 (pCGR-7), classifies the cDNA MA648 from *M. alpina* as the $\Delta 12$ -
20 desaturase.

Table 2
Fatty Acid as a Percentage of Total Lipid Extracted from Yeast

Plasmid in Yeast (enzyme)	18:2 Incorporated	α -18:3 Produced	γ -18:3 Produced	20:3 Incorporated	20:4 Produced	18:1* Present	18:2 Produced
pYES2 (control)	66.9	0	0	58.4	0	4	0
pCGR-2 (Δ 15)	60.1	5.7	0	50.4	0	0.7	0
pCGR-4 (Δ 5)	67	0	0	32.3	5.8	0.8	0
pCGR-5 (Δ 6)	62.4	0	4.0	49.9	0	2.4	0
pCGR-7 (Δ 12)	65.6	0	0	45.7	0	7.1	12.2

100 μ M substrate added

* 18:1 is an endogenous fatty acid in yeast

Key To Tables

18:1 =oleic acid
 18:2 =linoleic acid
 α -18:3 = α -linolenic acid
 γ -18:3 = γ -linolenic acid
 18:4 =stearidonic acid
 20:3 =dihomo- γ -linolenic acid
 20:4 =arachidonic acid

Example 7

Expression of $\Delta 5$ Desaturase in Plants

Expression in Leaves

This experiment was designed to determine whether leaves expressing
5 Ma29 (as determined by Northern) were able to convert exogenously applied
DGLA (20:3) to ARA (20:4).

The Ma29 desaturase cDNA was modified by PCR to introduce
convenient restriction sites for cloning. The desaturase coding region has been
inserted into a d35 cassette under the control of the double 35S promoter for
10 expression in *Brassica* leaves (pCGN5525) following standard protocols (*see*
USPN 5,424,200 and USPN 5,106,739). Transgenic *Brassica* plants containing
pCGN5525 were generated following standard protocols (*see* USPN 5,188,958
and USPN 5,463,174).

In the first experiment, three plants were used: a control, LPO04-1, and
15 two transgenics,, 5525-23 and 5525-29. LP004 is a low-linolenic *Brassica*
variety. Leaves of each were selected for one of three treatments: water, GLA
or DGLA. GLA and DGLA were purchased as sodium salts from NuChek Prep
and dissolved in water at 1 mg/ml. Aliquots were capped under N₂ and stored at
-70 degrees C. Leaves were treated by applying a 50 μ l drop to the upper
20 surface and gently spreading with a gloved finger to cover the entire surface.
Applications were made approximately 30 minutes before the end of the light
cycle to minimize any photo-oxidation of the applied fatty acids. After 6 days
of treatment one leaf from each treatment was harvested and cut in half through
the mid rib. One half was washed with water to attempt to remove
25 unincorporated fatty acid. Leaf samples were lyophilized overnight, and fatty
acid composition determined by gas chromatography (GC). The results are
shown in Table 3.

Table 3
Fatty Acid Analysis of Leaves from Ma29 Transgenic *Brassica* Plants

Treatment	SPL	16:00	16:01	18:00	18:01	18:10	18:1v	18:02	18:3g	18:03	18:04	20:00	20:01
	#	%	%	%	%	%	%	%	%	%	%	%	%
Water	33	12.95	0.08	2.63	2.51	1.54	0.98	16.76	0	45.52	0	0.09	0
	34	13.00	0.09	2.67	2.56	1.55	1.00	16.86	0	44.59	0	0.15	0
	35	14.13	0.09	2.37	2.15	1.27	0.87	16.71	0	49.91	0	0.05	0.01
	36	13.92	0.08	2.32	2.07	1.21	0.86	16.16	0	50.25	0	0.05	0
GLA	37	13.79	0.11	2.10	2.12	1.26	0.86	15.90	0.08	46.29	0	0.54	0.01
	38	12.80	0.09	1.94	2.08	1.35	0.73	14.54	0.11	45.61	0	0.49	0.01
	39	12.10	0.09	2.37	2.10	1.29	0.82	14.85	1.63	43.66	0	0.53	0
	40	12.78	0.10	2.34	2.22	1.36	0.86	15.29	1.72	47.22	0	0.50	0.02
	41	13.71	0.07	2.68	2.16	1.34	0.82	15.92	2.12	46.55	0	0.09	0
	42	14.10	0.07	2.75	2.35	1.51	0.84	16.66	1.56	46.41	0	0.09	0.01
	43	13.62	0.09	2.22	1.94	1.21	0.73	14.68	2.42	46.69	0	0.51	0.01
	44	13.92	0.09	2.20	2.17	1.32	0.85	15.22	2.30	46.05	0	0.53	0.02
DGLA	45	12.45	0.14	2.30	2.28	1.37	0.91	15.65	0.07	44.62	0	0.12	0.01
	46	12.67	0.15	2.69	2.50	1.58	0.92	15.96	0.09	42.77	0	0.56	0.01
	47	12.56	0.23	3.40	1.98	1.13	0.86	13.57	0.03	45.52	0	0.51	0.01
	48	13.07	0.24	3.60	2.51	1.63	0.88	13.54	0.04	45.13	0	0.50	0.01
	49	13.26	0.07	2.81	2.34	1.67	0.67	16.04	0.04	43.89	0	0.59	0
	50	13.53	0.07	2.84	2.41	1.70	0.70	16.07	0.02	44.90	0	0.60	0.01

Table 3 - Continued
Fatty Acid Analysis of Leaves from Ma29 Transgenic *Brassica* Plants

Treatment	SPL	20:02	20:03	20:04	20:05	22:00	22:01	22:02	22:03	22:06	24:0	24:1
	#	%	%	%	%	%	%	%	%	%	%	%
Water	33	0	0	0.29	0	0.01	0.09	16.26	0	0	0.38	0.18
	34	0.01	0	0.26	0	0.14	0.10	16.82	0.02	0.05	0.36	0.27
	35	0.01	0	0.25	0	0.12	0.06	11.29	0.04	0.05	0.29	0.25
	36	0	0.01	0.26	0	0.07	0.04	11.82	0.03	0.36	0.28	0.21
	37	0.02	0	0.21	0	0.18	0.08	15.87	0.06	0.20	0.30	0.17
	38	0.01	0	0.24	0	0.15	0.07	13.64	0.09	0.08	5.89	0.23
GLA	39	0.02	0.01	0.27	0	0.10	0.08	16.25	3.42	0.19	0.37	0.17
	40	0.01	0	0.27	0	0.10	0.10	14.74	0.05	0.10	0.36	0.14
	41	0	0	0.27	0	0.20	0.10	13.15	0.13	0.29	0.33	0.20
	42	0	0	0.28	0	0.11	0.11	12.60	0.02	0.24	0.38	0.13
	43	0.01	0	0.28	0	0.10	0.03	14.73	0.01	0.24	0.34	0.14
	44	0.02	0	0.26	0	0.13	0.07	14.43	0.05	0.16	0.33	0.17
DGLA	45	0.06	1.21	0.26	0	0.07	0.07	18.67	0.02	0.21	0.36	0.13
	46	0	1.94	0.27	0	0.11	0.09	17.97	0.09	0.39	0.41	0.11
	47	0.01	0.69	0.96	0	0.11	0.07	17.96	0	0.22	0.49	0.20
	48	0.01	0.70	0.74	0	0.14	0.09	17.14	0.05	0.32	0.52	0.10
	49	0	0.35	1.11	0	0.10	0.07	17.26	0.07	0.23	0.39	0.18
	50	0	0.20	0.87	0	0.21	0.07	15.73	0.04	0.15	0.37	0.18

Leaves treated with GLA contained from 1.56 to 2.4 wt% GLA. The fatty acid analysis showed that the lipid composition of control and transgenic leaves was essentially the same. Leaves of control plants treated with DGLA contained 1.2-1.9 w% DGLA and background amounts of ARA (.26-.27 wt%).

- 5 Transgenic leaves contained only .2-.7 wt% DGLA, but levels of ARA were increased (.74-1.1 wt%) indicating that the DGLA was converted to ARA in these leaves.

Expression in Seed

- 10 The purpose of this experiment was to determine whether a construct with the seed specific napin promoter would enable expression in seed.

The Ma29 cDNA was modified by PCR to introduce *XhoI* cloning sites upstream and downstream of the start and stop codons, respectively, using the following primers:

Madxho-forward:

- 15 5'-CUACUACUACUACTCGAGCAAGATGGGAACGGACCAAGG
(SEQ ID NO:25)

Madxho-reverse:

5'-CAUCAUCAUCAUCTCGAGCTACTCTTCCTTGGGACGGAG
(SEQ ID NO:26).

- 20 The PCR product was subcloned into pAMP1 (GIBCOBRL) using the CloneAmp system (GIBCOBRL) to create pCGN5522 and the $\Delta 5$ desaturase sequence was verified by sequencing of both strands.

- 25 For seed-specific expression, the Ma29 coding region was cut out of pCGN5522 as an *XhoI* fragment and inserted into the *SalI* site of the napin expression cassette, pCGN3223, to create pCGN5528. The *HindIII* fragment of pCGN5528 containing the napin 5' regulatory region, the Ma29 coding region, and the napin 3' regulatory region was inserted into the *HindIII* site of pCGN1557 to create pCGN5531. Two copies of the napin transcriptional unit were inserted in tandem. This tandem construct can permit higher expression of

the desaturases per genetic loci. pCGN5531 was introduced into *Brassica napus* cv.LP004 via Agrobacterium mediated transformation.

The fatty acid composition of twenty-seed pools of mature T2 seeds was analyzed by GC. Table 4 shows the results obtained with independent
5 transformed lines as compared to non-transformed LP004 seed. The transgenic seeds containing pCGN5531 contain two fatty acids that are not present in the control seeds, tentatively identified as taxoleic acid (5,9-18:2) and pinolenic acid (5,9,12-18:3), based on their elution relative to oleic and linoleic acid. These would be the expected products of $\Delta 5$ desaturation of oleic and linoleic
10 acids. No other differences in fatty acid composition were observed in the transgenic seeds.

Table 4
Composition of T2 Pooled Seed

	16:0	16:1	18:0	18:1	(5,9)18:2	18:2	(5,9,12)18:3	18:3	20:0	20:1	20:2	22:0	22:1	24:0
	%	%	%	%	%	%	%	%	%	%	%	%	%	%
LP004 control	3.86	0.15	3.05	69.1	0	18.51	0.01	1.65	1.09	1.40	0.03	0.63	0.05	0.42
5531-1	4.26	0.15	3.23	62.33	4.07	21.44	0.33	1.38	0.91	1.04	0.05	0.41	0.03	0.27
5531-2	3.78	0.14	3.37	66.18	4.57	17.31	0.27	1.30	1.03	1.18	0	0.47	0.01	0.30
5531-6	3.78	0.13	3.47	63.61	6.21	17.97	0.38	1.34	1.04	1.14	0.05	0.49	0.02	0.26
5531-10	3.96	0.17	3.28	63.82	5.41	18.58	0.32	1.43	0.98	1.11	0.02	0.50	0	0.31
5531-16	3.91	0.17	3.33	64.31	5.03	18.98	0.33	1.39	0.96	1.11	0	0.44	0	0
5531-28	3.81	0.13	2.58	62.64	5.36	20.95	0.45	1.39	0.83	1.15	0.01	0.36	0.05	0.21

Northern analysis is performed on plants to identify those expressing Ma29. Developing embryos are isolated approximately 25 days post anthesis or when the napin promoter is induced, and floated in a solution containing GLA or DGLA as described in Example 7. Fatty acid analysis of the embryos is then performed by GC to determine the amount of conversion of DGLA to ARA, following the protocol adapted for leaves in Example 7. The amount of ARA incorporated into triglycerides by endogenous *Brassica* acyltransferases is then evaluated by GC analysis as in Example 7.

Example 8

Expression of *M. alpina* $\Delta 6$ Desaturase in *Brassica napus*

The Ma524 cDNA was modified by PCR to introduce cloning sites using the following primers:

Ma524PCR-1 (SEQ ID NO:27)

5'-CUACUACUACUATCTAGACTCGAGACCATGGCTGCTGCT
CCAGTGTG

Ma524PCR-2 (SEQ ID NO:28)

5'-CAUCAUCAUCAUAGGCCTCGAGTTACTGCGCCTTACCCAT

These primers allowed the amplification of the entire coding region and added *Xba*I and *Xho*I sites to the 5'-end and *Xho*I and *Stu*I sites to the 3' end. The PCR product was subcloned into pAMP1 (GIBCOBRL) using the CloneAmp system (GIBCOBRL) to create pCGN5535 and the $\Delta 6$ desaturase sequence was verified by sequencing of both strands.

For seed-specific expression, the Ma524 coding region was cut out of pCGN5535 as an *Xho*I fragment and inserted into the *Sal*I site of the napin expression cassette, pCGN3223, to create pCGN5536. The *Not*I fragment of pCGN5536 containing the napin 5' regulatory region, the Ma524 coding region, and the napin 3' regulatory region was inserted into the *Not*I site of pCGN1557

to create pCGN5538. pCGN5538 was introduced into *Brassica napus* cv.LP004 via Agrobacterium mediated transformation.

Maturing T2 seeds were collected from 6 independent transformation events in the greenhouse. The fatty acid composition of single seeds was
5 analyzed by GC. Table 5 shows the results of control LP004 seeds and six 5538 lines. All of the 5538 lines except #8 produced seeds containing GLA. Presence of GLA segregated in these seeds as is expected for the T2 selfed seed population. In addition to GLA, the *M. alpina* $\Delta 6$ desaturase is capable of producing 18:4 (stearidonic) and another fatty acid believed to be the 6,9-18:2.

10 The above results show that desaturases with three different substrate specificities can be expressed in a heterologous system and used to produce poly-unsaturated long chain fatty acids. Exemplified were the production of ARA (20:4) from the precursor 20:3 (DGLA), the production of GLA (18:3) from 18:2 substrate, and the conversion of 18:1 substrate to 18:2, which is the
15 precursor for GLA.

Table 5
Fatty Acid Analysis of Seeds from Ma524 Transgenic *Brassica* Plants

SPL #	16:0	16:1	18:0	18:1	6,9	18:2	18:2	18:3ga	18:3	18:4	20:1	22:0	22:1	24:0	24:1
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
LPO04-1	4.33	0.21	3.78	72.49	0	13.97	0	1.7	0	1.34	0.71	0.02	0.58	0.27	
-2	4.01	0.16	3.09	73.59	0	14.36	0.01	1.4	0	1.43	0.66	0.02	0.5	0.2	
-3	4.12	0.19	3.56	70.25	0	17.28	0	1.57	0	1.28	0.5	0.02	0.39	0.2	
-4	4.22	0.2	2.7	70.25	0	17.86	0	1.61	0	1.31	0.53	0.02	0.4	0.24	
-5	4.02	0.16	3.41	72.91	0	14.45	0.01	1.45	0	1.37	0.7	0.02	0.51	0.26	
-6	4.22	0.18	3.23	71.47	0	15.92	0.01	1.52	0	1.32	0.69	0.02	0.51	0.27	
-7	4.1	0.16	3.47	72.06	0	15.23	0	1.52	0	1.32	0.63	0.03	0.49	0.23	
-9	4.01	0.17	3.71	72.98	0	13.97	0.01	1.41	0	1.45	0.74	0.03	0.58	0.23	
-10	4.04	0.16	3.57	70.03	0	17.46	0	1.5	0	1.33	0.61	0.03	0.36	0.24	
5538-1-1	4.61	0.2	3.48	68.12	1.37	10.68	7.48	1.04	0.33	1.19	0.49	0.02	0.33	0.13	
-2	4.61	0.22	3.46	68.84	1.36	10.28	7.04	1.01	0.31	1.15	0.48	0.02	0.39	0	
-3	4.78	0.24	3.24	65.86	0	21.36	0	1.49	0	1.08	0.46	0.02	0.38	0.22	
-4	4.84	0.3	3.89	67.64	1.67	9.9	6.97	1.02	0.36	1.14	0.53	0.02	0.5	0.18	
-5	4.64	0.2	3.58	64.5	3.61	8.85	10.14	0.95	0.48	1.19	0.47	0.01	0.33	0.12	
-6	4.91	0.27	3.44	66.51	1.48	11.14	7.74	1.15	0.33	1.08	0.49	0.02	0.34	0.13	
-7	4.87	0.22	3.24	65.78	1.27	11.92	8.38	1.2	0	1.12	0.47	0.02	0.37	0.16	

Table 5
Fatty Acid Analysis of Seeds from Ma524 Transgenic *Brassica* Plants

SPL #	16:0	16:1	18:0	18:1	6,9	18:2	18:2	18:3ga	18:3	18:4	20:1	22:0	22:1	24:0	24:1
%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
-8	4.59	0.22	3.4	70.77	0	16.71	0	1.35	0	1.14	0.48	0.02	0.39	0.15	
-9	4.63	0.23	3.51	69.66	2.01	8.77	7.24	0.97	0	1.18	0.52	0.02	0.3	0.11	
-10	4.56	0.19	3.55	70.68	0	16.89	0	1.37	0	1.22	0.54	0.02	0.22	0.03	
5538-3-1	4.74	0.21	3.43	67.52	1.29	10.91	7.77	1.03	0.28	1.11	0.5	0.02	0.35	0.14	
-2	4.72	0.21	3.24	67.42	1.63	10.37	8.4	0.99	0	1.12	0.49	0.02	0.36	0.15	
-3	4.24	0.21	3.52	71.31	0	16.53	0	1.33	0	1.12	0.45	0.02	0.4	0.14	
-4	4.64	0.21	3.45	67.92	1.65	9.91	7.97	0.91	0.33	1.14	0.47	0.02	0.37	0.14	
-5	4.91	0.25	3.31	67.19	0	19.92	0.01	1.39	0	1.05	0.48	0.02	0.37	0.14	
-6	4.67	0.21	3.25	67.07	1.23	11.32	8.35	0.99	0	1.16	0.47	0.02	0.33	0.16	
-7	4.53	0.19	2.94	64.8	4.94	8.45	9.95	0.93	0.44	1.13	0.37	0.01	0.27	0.12	
-8	4.66	0.22	3.68	67.33	0.71	12	6.99	1.1	0.24	1.18	0.48	0.03	0.36	0.17	
-9	4.65	0.24	3.11	67.42	0.64	12.71	6.93	1.16	0.25	1.08	0.45	0.02	0.32	0.17	
-10	4.88	0.27	3.33	65.75	0.86	12.89	7.7	1.1	0.24	1.08	0.46	0.01	0.34	0.16	
5538-4-1	4.65	0.24	3.8	62.41	0	24.68	0	1.6	0.01	0.99	0.45	0.02	0.33	0.13	
-2	5.37	0.31	3	57.98	0.38	18.04	10.5	1.41	0	0.99	0.48	0.02	0.3	0.19	
-3	4.61	0.22	3.07	63.62	0.3	16.46	7.67	1.2	0	1.18	0.45	0.02	0.29	0.14	

Table 5
Fatty Acid Analysis of Seeds from Ma524 Transgenic *Brassica* Plants

SPL #	16:0	16:1	18:0	18:1	6,9	18:2	18:2	18:3ga	18:3	18:4	20:1	22:0	22:1	24:0	24:1
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
-4	4.39	0.19	2.93	65.97	0	22.36	0	1.45	0	1.17	0.41	0.03	0.32	0.15	
-5	5.22	0.29	3.85	62.1	2.35	10.25	11.39	0.93	0.41	1.04	0.6	0.02	0.47	0.17	
-6	4.66	0.18	2.85	66.79	0.5	13.03	7.66	0.97	0.22	1.28	0.42	0.02	0.31	0.14	
-7	4.85	0.26	3.03	57.43	0.26	28.04	0.01	2.59	0.01	1.13	0.56	0.02	0.4	0.23	
-8	5.43	0.28	2.94	54.8	1.84	13.79	15.67	1.36	0.53	1.1	0.55	0.02	0.35	0.19	
-9	4.88	0.24	3.32	62.3	0.58	14.86	9.04	1.34	0.29	1.13	0.52	0.02	0.37	0.19	
-10	4.53	0.2	2.73	64.2	0.07	24.15	0	1.52	0	1.09	0.39	0.02	0.27	0.17	
5538-5-1	4.5	0.15	3.35	66.71	0.88	11.7	8.38	1.04	0.3	1.24	0.49	0.02	0.29	0.17	
-2	4.77	0.23	3.06	62.67	0.68	15.2	8.8	1.31	0.28	1.15	0.46	0.02	0.3	0.19	
-3	4.59	0.22	3.61	64.35	2.29	9.95	10.57	1.01	0.45	1.21	0.48	0.02	0.26	0.16	
-4	4.86	0.26	3.4	67.69	0.65	12.24	6.61	1.09	0.23	1.07	0.45	0.02	0.32	0.14	
-5	4.49	0.21	3.3	69.25	0.04	16.51	2.18	1.2	0	1.11	0.44	0.02	0.33	0.16	
-6	4.5	0.21	3.47	70.48	0.08	14.9	2.19	1.22	0	1.13	0.49	0.02	0.33	0.16	
-7	4.39	0.21	3.44	67.59	2.38	9.24	8.98	0.89	0	1.18	0.44	0.02	0.28	0.14	
-8	4.52	0.22	3.17	68.33	0.01	18.91	0.73	1.32	0.01	1.08	0.45	0.02	0.29	0.17	
-9	4.68	0.2	3.05	64.03	1.93	11.03	11.41	1.02	0.01	1.15	0.39	0.02	0.21	0.15	

Table 5
Fatty Acid Analysis of Seeds from Ma524 Transgenic *Brassica* Plants

SPL #	16:0	16:1	18:0	18:1	6,9	18:2	18:2	18:3ga	18:3	18:4	20:1	22:0	22:1	24:0	24:1
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
-10	4.57	0.2	3.1	67.21	0.61	12.62	7.68	1.07	0.25	1.14	0.43	0.02	0.25	0.15	
5538-8-1	4.95	0.26	3.14	64.04	0	23.38	0	1.54	0	0.99	0.42	0.02	0.38	0.17	
-2	4.91	0.26	3.71	62.33	0	23.97	0	1.77	0	0.95	0.53	0.02	0.42	0.19	
-3	4.73	0.25	4.04	63.83	0	22.36	0.01	1.73	0	1.05	0.55	0.02	0.45	0.16	
-4	5.1	0.35	3.8	60.45	0	24.45	0.01	2.13	0	1.07	0.65	0.03	0.53	0.24	
-5	4.98	0.3	3.91	62.48	0	23.44	0	1.77	0	1.01	0.51	0.01	0.43	0.21	
-6	4.62	0.21	3.99	66.14	0	20.38	0	1.48	0	1.15	0.53	0.02	0.48	0.19	
-7	4.64	0.22	3.55	64.6	0	22.65	0	1.38	0	1.09	0.45	0.02	0.41	0.19	
-8	5.65	0.38	3.18	56.6	0	30.83	0.02	0.02	0	0.98	0.55	0.03	0.39	0.26	
-9	8.53	0.63	6.9	51.76	0	26.01	0	0.01	0	1.41	1.21	0.07	0.96	0.33	
-10	5.52	0.4	3.97	57.92	0	28.95	0	0.02	0	0.95	0.52	0.02	0.41	0.16	
5538-10-1	4.44	0.19	3.5	68.42	0	19.51	0	1.32	0	1.14	0.45	0.02	0.31	0.16	
-2	4.57	0.21	3.07	66.08	0	21.99	0.01	1.36	0	1.12	0.41	0.02	0.31	0.16	
-3	4.63	0.21	3.48	67.43	0	20.27	0.01	1.32	0	1.12	0.46	0.02	0.21	0.08	
-4	4.69	0.19	3.22	64.62	0	23.16	0	1.35	0	1.08	0.46	0.02	0.33	0.2	
-5	4.58	0.2	3.4	68.75	0	20.17	0.01	0.02	0	1.1	0.45	0.02	0.34	0.17	

Table 5
Fatty Acid Analysis of Seeds from Ma524 Transgenic *Brassica* Plants

SPL #	16:0	16:1	18:0	18:1	6,9	18:2	18:2	18:3ga	18:3	18:4	20:1	22:0	22:1	24:0	24:1
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
-8	4.55	0.21	0	73.55	0.05	14.91	2.76	1.21	0.07	1.24	0.51	0.02	0.19	0	
-9	4.58	0.21	3.28	66.19	0	21.55	0	1.35	0	1.12	0.43	0.02	0.33	0.16	
-10	4.52	0.2	3.4	68.37	0	19.33	0.01	1.3	0	1.13	0.46	0.02	0.35	0.18	

Example 9

Expression of *M. alpina* $\Delta 12$ desaturase in *Brassica napus*

The Ma648 cDNA was modified by PCR to introduce cloning sites using the following primers:

- 5 Ma648PCR-for (SEQ ID NO:29)
 5'-CUACUACUACUAGGATCCATGGCACCTCCCAACACT
 Ma648PCR-rev (SEQ ID NO:30)
 5'-CAUCAUCAUCAUGGTACCTCGAGTTACTTCTTGAAAAAGAC

10 These primers allowed the amplification of the entire coding region and added a BamHI site to the 5' end and KpnI and XhoI sites to the 3' end. The PCR product was subcloned into pAMP1 (GIBCOBRL) using the CloneAmp system (GIBCOBRL) to create pCGN5540 and the $\Delta 12$ desaturase sequence was verified by sequencing of both strands.

15 For seed-specific expression, the Ma648 coding region was cut out of pCGN5540 as a BamHI/XhoI fragment and inserted between the BglII and XhoI sites of the napin expression cassette, pCGN3223, to create pCGN5542. The Asp718 fragment of pCGN5541 containing the napin 5' regulatory region, the Ma648 coding region, and the napin 3' regulatory region was inserted into the Asp718 site of pCGN5138 to create pCGN5542. pCGN5542 was
20 introduced into two varieties of *Brassica napus* via *Agrobacterium* mediated transformation. The commercial canola variety, SP30021, and a low-linolenic line, LP30108 were used.

25 Mature selfed T2 seeds were collected from 19 independent LP30108 transformation events and a non-transformed control grown in the greenhouse. These seeds are expected to be segregating for the $\Delta 12$ desaturase transgene. The fatty acid composition of 20-seed pools was analyzed by GC. The results are shown in Table 6. All transformed lines contained increased levels of 18:2, the product of the $\Delta 12$ desaturase. Levels of 18:3 were not significantly increased in these plants. Events # 11 and 16 showed the greatest accumulation

of 18:2 in the pooled seeds. To investigate the segregation of 18:2 levels in the T2 seeds and to identify individual plants to be taken on to subsequent generations, half-seed analysis was done. Seeds were germinated overnight in the dark at 30 degrees on water-soaked filter paper. The outer cotyledon was excised for GC analysis and the rest of the seedling was planted in soil. Results of some of these analyses are shown in Table 7. Individual T2 seeds containing the *M. alpina* $\Delta 12$ desaturase accumulated up to 60% 18:2 in the seeds. Sample 97xx1116 #59 is an example of a null segregant. Even in the highest 18:2 accumulators, levels of 18:3 were increased only slightly. These and other individually selected T2 plants were grown in the greenhouse and in the field to produce T3 seed.

Mature selfed T2 seeds were collected from 20 independent SP30021 transformation events and a non-transformed control grown in the greenhouse. These seeds are expected to be segregating for the $\Delta 12$ desaturase transgene. The fatty acid composition of 20-seed pools was analyzed by GC. The data are presented in Table 8. All transformed lines contained increased levels of 18:2, the product of the $\Delta 12$ desaturase. As in the low-linolenic LP30108 line, levels of 18:3 were not significantly increased. Events # 4 and 12 showed the greatest accumulation of 18:2 in the pooled seeds. To investigate the segregation of 18:2 levels in the T2 seeds and to identify individual plants to be taken on to subsequent generations, alf-seed analysis was done. Seeds were germinated overnight in the dark at 30 degrees on water-soaked filter paper. The outer cotyledon was excised for GC analysis and the rest of the seedling was planted in soil. Results of some of these analyses are shown in Table 9. Samples 97xx1157 #88 and #18 are examples of null segregants for 5542-SP30021-4 and 5542-SP30021-12 respectively. These and other individually selected T2 plants were grown in the greenhouse and in the field to produce T3 seed

Table 6

CYCLE ID	SPL NO	STRAIN ID	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0
97XX1098	45	5542-LP30108-16	7.04	0.43	1.12	18.01	66.36	4.76	0.5	0.84	0.3	0.44
97XX1098	22	5542-LP30108-16	5.17	0.29	2.11	22.01	65.18	3.15	0.63	0.75	0.21	0.36
97XX1098	40	5542-LP30108-16	4.99	0.2	2.05	23.91	63.13	3.3	0.73	0.85	0.23	0.49
97XX1098	28	5542-LP30108-16	4.47	0.19	1.75	26.7	62.39	2.46	0.58	0.85	0.2	0.32
97XX1098	2	5542-LP30108-16	4.54	0.21	1.66	26.83	61.89	2.9	0.55	0.82	0.18	0.33
97XX1098	58	5542-LP30108-16	6.05	0.31	1.36	24.11	61.36	3.8	0.72	1.13	0.26	0.58
97XX1098	83	5542-LP30108-16	5.13	0.17	2.03	27.05	60.93	2.62	0.7	0.71	0.14	0.4
97XX1098	34	5542-LP30108-16	4.12	0.19	1.44	29.35	60.54	2.53	0.43	0.89	0.17	0.25
97XX1116	37	5542-LP30108-11	4	0.14	2.43	23.29	63.99	2.6	0.58	0.69	0.71	1.11
97XX1116	88	5542-LP30108-11	3.8	0.18	2.04	23.59	63.93	2.95	0.54	0.81	0.99	0.82
97XX1116	36	5542-LP30108-11	4.15	0.2	1.51	25.94	62.14	2.74	0.47	0.87	0.79	0.81
97XX1116	31	5542-LP30108-11	6.29	0.35	1.04	24.14	60.91	4.02	0.55	0.91	0.75	0.72
97XX1116	10	5542-LP30108-11	6.97	0.4	3.36	18.9	60.66	4.68	1.2	0.7	0.53	1.71
97XX1116	32	5542-LP30108-11	3.96	0.16	2.61	26.73	60.54	3.38	0.66	0.87	0.2	0.62
97XX1116	55	5542-LP30108-11	4.26	0.22	0.98	28.57	59.94	3.24	0.4	0.68	0.71	0.75
97XX1116	12	5542-LP30108-11	4.17	0.23	1.42	28.61	59.52	3.26	0.51	0.95	0.29	0.67

Table 6

CYCLE ID	SPL NO	STRAIN ID	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0
97XX1116	86	5542-LP30108-11	4.23	0.3	1.09	28.34	59.2	3.95	0.48	0.91	0.55	0.71
97XX1116	61	5542-LP30108-11	4.13	0.16	1.92	30.18	58.67	2.65	0.56	0.88	0.25	0.41
97XX1116	60	5542-LP30108-11	4.42	0.26	1.61	28.77	58.6	3.26	0.53	0.85	0.68	0.75
97XX1116	91	5542-LP30108-11	7.82	0.67	2.37	17.97	58.43	4.85	0.94	0.86	3.87	1.71
97XX1116	59	5542-LP30108-11	3.56	0.2	1.6	65.5	23.03	2.23	0.52	1.54	0.49	0.69

Table 7

	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0
%	%	%	%	%	%	%	%	%	%	%
5542-LP30108-1	4.6	0.15	1.93	50.44	38.54	2.06	0.65	1.11	0.09	0.37
5542-LP30108-2	4.63	0.17	1.78	41.11	47.53	2.46	0.62	1.02	0.14	0.38
5542-LP30108-3	4.96	0.18	2.07	48.16	40.01	2.17	0.73	1.13	0.1	0.39
5542-LP30108-4	4.36	0.15	1.94	46.51	42.57	1.95	0.64	1.06	0.11	0.35
5542-LP30108-5	4.45	0.14	2.19	49.54	39.13	2.14	0.72	1.14	0.11	0.38
5542-LP30108-6	4.97	0.16	1.86	49.23	39.2	2.17	0.7	1.12	0.11	0.41
5542-LP30108-7	4.46	0.13	2.72	39.6	48.65	2.02	0.81	0.96	0.13	0.4
5542-LP30108-8	4.63	0.18	1.78	47.86	41	2.31	0.62	1.09	0.11	0.36
5542-LP30108-9	4.64	0.16	1.75	42.5	46.57	2.2	0.61	1	0.13	0.35
5542-LP30108-10	4.46	0.15	2.37	43.61	45.29	1.77	0.71	1.02	0.12	0.36
5542-LP30108-11	4.58	0.25	1.88	37.08	50.95	2.94	0.64	0.96	0.16	0.42
5542-LP30108-12	4.46	0.18	1.69	43.62	45.36	2.44	0.59	1.09	0.14	0.34
5542-LP30108-13	4.45	0.15	2.33	51	37.71	1.91	0.75	1.12	0.09	0.4
5542-LP30108-14	4.3	0.16	2.04	45.93	42.78	2.46	0.66	1.07	0.14	0.37
5542-LP30108-15	4.18	0.16	2.17	43.79	45.2	2.14	0.68	1.04	0.15	0.36
5542-LP30108-16	5.04	0.18	1.89	32.32	55.78	2.68	0.63	0.84	0.2	0.36

Table 7

	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0
	%	%	%	%	%	%	%	%	%	%
5542-LP30108-18	4.2	0.14	2.23	50.63	38.51	1.79	0.72	1.15	0.1	0.37
5542-LP30108-19	4.63	0.18	1.81	52.51	36.26	2.12	0.68	1.19	0.1	0.4
5542-LP30108-20	4.77	0.15	2.78	39.76	48.06	2.25	0.75	0.91	0.13	0.36
LP30108 control	4.31	0.22	2.05	66.15	22.59	1.87	0.77	1.3	0.07	0.44

Table 8

STRAIN ID	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0
5542-SP30021-1	4.37	0.17	2.17	40.26	39.43	11.06	0.74	1.14	0.14	0.42
5542-SP30021-2	4.33	0.18	1.51	43.07	36.03	12.57	0.57	1.21	0.14	0.33
5542-SP30021-3	5.2	0.22	3.1	43.7	37.04	8.03	0.92	1.06	0.13	0.48
5542-SP30021-4	4.37	0.15	1.94	34.26	45.12	12.04	0.6	0.96	0.17	0.3
5542-SP30021-5	4.15	0.17	1.73	48.98	31.13	11.41	0.63	1.26	0.13	0.35
5542-SP30021-6	4.52	0.17	1.92	38.1	42.39	10.53	0.67	1.04	0.18	0.39
5542-SP30021-7	4.58	0.18	1.66	41.87	37.52	11.8	0.62	1.14	0.15	0.36
5542-SP30021-8	4.46	0.17	1.59	42.69	36.93	11.88	0.59	1.14	0.14	0.35
5542-SP30021-9	4.63	0.19	1.69	39.89	39.75	11.48	0.62	1.09	0.15	0.38
5542-SP30021-10	4.74	0.16	1.79	39.19	40.51	11.42	0.63	0.99	0.13	0.34
5542-SP30021-11	4.57	0.16	1.71	38.13	42	11.15	0.62	1.04	0.18	0.36
5542-SP30021-12	4.05	0.16	2.04	35.44	43.47	12.45	0.62	1.07	0.21	0.33
5542-SP30021-13	4.37	0.15	1.79	38.74	41.28	11.36	0.62	1.04	0.16	0.35
5542-SP30021-14	4.32	0.16	1.47	42.32	37.17	12.3	0.54	1.16	0.16	0.32
5542-SP30021-15	4.25	0.18	1.65	44.96	34.28	12.39	0.59	1.13	0.14	0.32

Table 8

STRAIN ID	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0
5542-SP30021-16	4.53	0.17	1.91	42.13	38.32	10.51	0.67	1.12	0.14	0.38
5542-SP30021-17	4.16	0.19	1.7	50.65	29.3	11.4	0.61	1.29	0.11	0.36
5542-SP30021-18	4.24	0.17	1.68	44.47	35.46	11.52	0.6	1.19	0.14	0.34
5542-SP30021-19	4.1	0.18	1.8	46.67	33.87	10.86	0.63	1.24	0.13	0.37
5542-SP30021-20	4.3	0.17	1.64	39.6	40.39	11.53	0.57	1.12	0.16	0.32
SP30021	4.38	0.21	1.47	56.51	22.59	12.04	0.62	1.45	0.11	0.39

Table 9

CYCLE ID	SPL NO	STRAIN ID	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0
97XX1156	96	5542-SP30021-4	3.71	0.13	1.36	29.29	51.74	11.57	0.41	0.85	0.18	0.46
97XX1156	50	5542-SP30021-4	2.95	0.11	1.33	28.78	50.97	13.83	0.3	0.99	0.28	0.32
97XX1158	10	5542-SP30021-4	4.05	0.16	2.47	31.18	50.88	8.77	0.67	0.89	0.22	0.33
97XX1158	32	5542-SP30021-4	3.56	0.15	1.44	30.73	50.1	11.86	0.47	0.91	0.21	0.22
97XX1158	56	5542-SP30021-4	4.44	0.19	3.09	30.64	49.71	9.39	0.83	0.79	0.2	0.4
97XX1157	80	5542-SP30021-4	4.05	0.18	1.32	27.41	49.59	14.81	0.53	1.19	0.29	0.4
97XX1158	39	5542-SP30021-4	4.04	0.15	2.98	28.62	49.52	12.28	0.69	0.86	0.31	0.27
97XX1156	17	5542-SP30021-4	3.65	0.15	2.43	29.38	49.42	12.3	0.52	0.92	0.67	0.35
97XX1156	60	5542-SP30021-4	3.75	0.17	1.7	30.03	49.13	12.87	0.51	1.01	0.27	0.35
97XX1157	83	5542-SP30021-4	4.15	0.2	1.77	29.72	49.08	12.22	0.66	1.21	0.16	0.52
97XX1157	86	5542-SP30021-4	3.6	0.14	1.12	27.65	49.01	16.05	0.48	1.21	0.33	0.08
97XX1158	77	5542-SP30021-4	4.14	0.17	1.58	31.98	48.82	10.72	0.65	1	0.28	0.44
97XX1157	88	5542-SP30021-4	3.36	0.15	1.22	56.42	21.63	13.78	0.58	1.85	0.06	0.65

Table 9

CYCLE ID	SPL NO	STRAIN ID	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0
97XX1157	39	5542-SP30021-12	2.84	0.04	1.84	29.6	53.16	9.52	0.57	1.32	0.35	0.48
97XX1157	55	5542-SP30021-12	3.28	0.1	2.18	30.36	52.27	9.26	0.63	1.15	0.22	0.41
97XX1157	10	5542-SP30021-12	3.5	0.06	1.51	29.78	50.98	11.13	0.64	1.45	0.4	0.26
97XX1157	41	5542-SP30021-12	3.31	0.08	1.64	30.18	50.51	11.59	0.57	1.27	0.24	0.41
97XX1157	35	5542-SP30021-12	3.31	0.09	1.57	30.36	50.1	12.17	0.5	1.15	0.23	0.35
97XX1157	1	5542-SP30021-12	3.45	0.11	2.88	32.11	49.45	8.69	0.82	1.22	0.27	0.63
97XX1157	16	5542-SP30021-12	2.91	0.09	1.52	29.35	48.88	14.26	0.58	1.39	0.15	0.3
97XX1157	50	5542-SP30021-12	3.29	0.09	2.13	33.23	48.78	9.87	0.67	1.06	0.18	0.47
97XX1157	25	5542-SP30021-12	2.83	0.05	1.4	33.22	48.52	11.22	0.5	1.33	0.26	0.42
97XX1157	57	5542-SP30021-12	2.94	0.13	1.46	32.85	47.58	12.21	0.57	1.31	0.27	0.47
97XX1157	56	5542-SP30021-12	3.01	0.07	1.63	31.53	47	14.02	0.59	1.31	0.28	0.23
97XX1157	6	5542-SP30021-12	3.9	0.13	1.5	32.43	46.98	12.45	0.52	1.11	0.21	0.49
97XX1157	18	5542-SP30021-12	3.88	0.16	1.73	57.94	22.33	10.51	0.74	1.68	0.11	0.64

Example 10

Simultaneous expression of *M. alpina* $\Delta 6$ and $\Delta 12$ desaturases in *Brassica napus*

5 In order to express the *M. alpina* $\Delta 6$ and $\Delta 12$ desaturases from the same T-DNA, the following construct for seed-specific expression was made.

 The NotI fragment of pCGN5536 containing the napin 5' regulatory region, the Ma524 coding region, and the napin 3' regulatory region was inserted into the NotI site of pCGN5542 to create pCGN5544. The
10 expression modules were oriented in such a way that the direction of transcription from Ma524 and Ma648 and the nptII marker is the same.

 PCGN5544 was introduced into *Brassica napus* cv.LP30108 via *Agrobacterium* mediated transformation. Mature selfed T2 seeds were collected from 16 independent LP30108 transformation events and a non-transformed
15 control that were grown in the greenhouse. These seeds are expected to be segregating for the $\Delta 6 + \Delta 12$ desaturase transgene. The fatty acid composition of 20-seed pools was analyzed by GC. The results are presented in Table 10. All but one of the lines (5544-LP30108-3) shows an altered oil composition as compared to the controls. GLA was produced in all but three of the lines (-3, -4,
20 -11); two of the three without GLA (-4, -11) showed increased 18:2 indicative of expression of the $\Delta 12$ desaturase. As a group, the levels of GLA observed in plants containing the double $\Delta 6 + \Delta 12$ construct (pCGN5544) were higher than those of plants containing pCGN5538 ($\Delta 6$ alone). In addition, levels of the $\Delta^{6,9}$ 18:2 are much reduced in the plants containing the $\Delta 12 + \Delta 6$ as compared to $\Delta 6$
25 alone. Thus, the combination of $\Delta 6$ and $\Delta 12$ desaturases on one T-DNA leads to the accumulation of more GLA and fewer side products than expression of $\Delta 6$ desaturase alone. To investigate the segregation of GLA levels in the T2 seeds and to identify individual plants to be taken on to subsequent generations, half-seed analysis was done. Seeds were germinated overnight in the dark at 30
30 degrees on water-soaked filter paper. The outer cotyledon was excised for GC analysis and the rest of the seedling was planted in soil. Results of some of

these analyses are shown in Table 11. As expected for the T2 population, levels of GLA and 18:2 are segregating in the individual seeds. GLA content of up to 60% of total fatty acids was observed in individual seeds. Individual events were selected to be grown in the greenhouse and field for production of T3 seed.

Transgenic plants including *Brassica*, soybean, safflower, corn flax and sunflower expressing the constructs of this invention can be a good source of GLA.

Typical sources of GLA such as borage produce at most 25% GLA. In contrast the plants in Table 10 contain up to 30% GLA. Furthermore, the individual seeds shown in Table 11 contain up to 60% GLA.

Table 10

	16:0	16:1	18:0	18:1	18:2	18:2	18:2	18:3	18:3	18:4	20:0	20:1	22:0
						$\Delta 6,9$	$\Delta 9,12$	$\Delta 6,9,12$	$\Delta 9,12,15$				
	%	%	%	%	%	%	%	%	%	%	%	%	%
5544-LP30108-1	4.54	0.17	1.91	49.96	0	30.98	7.97	1.85	0.11	0.68	1.17	0.41	
5544-LP30108-2	4.69	0.19	2.15	38.49	0	33.94	16.21	1.73	0.25	0.72	0.96	0.41	
5544-LP30108-3	4.26	0.2	1.97	66.68	0	22.13	0.08	1.96	0.01	0.73	1.33	0.42	
5544-LP30108-4	4.59	0.24	1.76	44.21	0	44.54	0.02	2.19	0.01	0.62	1.08	0.4	
5544-LP30108-5	4.5	0.18	2.28	47.57	0	26.41	14.42	1.71	0.22	0.78	1.1	0.43	
5544-LP30108-6	4.51	0.16	2.12	31.95	0.01	26.94	29.8	1.41	0.5	0.81	1.02	0.51	
5544-LP30108-7	4.84	0.21	1.68	38.24	0	32.27	18.21	1.87	0.33	0.66	1.04	0.43	
5544-LP30108-10	5	0.28	1.86	41.17	0	46.54	0.36	2.58	0.02	0.6	0.91	0.37	
5544-LP30108-11	4.57	0.2	1.74	47.29	0	41.49	0.03	2.22	0.01	0.64	1.17	0.4	
5544-LP30108-12	4.87	0.18	2.65	34.53	0	30.37	23.12	1.46	0.36	0.83	0.95	0.45	
5544-LP30108-13	4.41	0.16	2.32	40.82	0.11	26.8	21.05	1.53	0.37	0.77	1.06	0.42	
5544-LP30108-14	4.38	0.2	2.21	29.91	0.16	28.01	30.62	1.46	0.59	0.76	0.97	0.47	
5544-LP30108-15	4.79	0.22	2.23	23.42	0.02	28.73	35.68	1.51	0.77	0.87	0.89	0.56	
5544-LP30108-16	4.54	0.18	1.78	40.81	0	35.24	12.83	1.95	0.27	0.68	1.02	0.43	
5544-LP30108-17	4.63	0.18	2.28	46.96	0	31.06	10.6	1.7	0.14	0.76	1.06	0.42	
5544-LP30108-20	4.87	0.29	1.44	31.81	0.15	23.51	32.85	1.64	0.69	0.89	0.96	0.67	

Table 10

16:0	16:1	18:0	18:1	18:2	18:2	18:2	18:3	18:3	18:4	20:0	20:1	22:0
						$\Delta 6,9$	$\Delta 9,12$	$\Delta 6,9,12$	$\Delta 9,12,15$			
%	%	%	%	%	%	%	%	%	%	%	%	%
<hr/>												
LP30108 control	3.89	0.25	1.19	67.73	0	22.46	0.1	1.97	0	0.54	1.32	0.44

Table 11

CYCLE ID	SPL NO	STRAIN ID	16:0	16:1	18:0	18:1	18:2_Δ6,9	18:2_Δ9,12	18:3_Δ6,9, 12	18:3_Δ9,12, 15	18:4	20:0	20:1
97XX1333	64	5544-LP30108-20	6.53	0.15	0.98	23.33	0.01	21.1	43.3	1.34	0.84	0.52	0.97
97XX1333	65	5544-LP30108-20	6.9	0.29	1.17	8.89	0.03	15.07	60.5	1.12	2.23	0.98	0.86
97XX1333	66	5544-LP30108-20	8.15	0.2	3.6	16.87	0.11	16.05	48.23	1.1	1.18	1.71	0.66
97XX1333	67	5544-LP30108-20	8.85	0.35	1.2	14.49	0.01	25.66	43.98	1.8	1.03	0.65	0.76
97XX1333	68	5544-LP30108-20	6.05	0.16	1.27	17.85	0.16	16.13	53.16	1.14	1.25	0.71	0.85
97XX1333	69	5544-LP30108-20	7.16	0.21	1.33	11.51	0.09	17.42	56.13	1.41	1.58	0.93	0.68
97XX1333	70	5544-LP30108-20	3.46	0.04	1.76	18.38	0.03	22.55	48.55	1.22	1.04	0.83	0.95
97XX1333	71	5544-LP30108-20	3.71	0.05	1.74	16.11	0.01	26.93	45.79	1.47	1.02	0.89	1
97XX1333	72	5544-LP30108-20	3.5	0.04	1.76	23.74	0.02	35.38	30.82	1.87	0.58	0.65	0.89
97XX1333	73	5544-LP30108-20	4.67	0.11	1.87	17.98	0.04	22.47	47.89	1.17	0.89	0.93	0.88
97XX1333	74	5544-LP30108-20	4.52	0.09	1.86	13.77	0.03	20.9	52.96	1.31	1.19	1.03	0.88
97XX1333	75	5544-LP30108-20	5.26	0.13	1.64	16.46	0.05	21.75	49.42	1.25	1.08	0.83	0.86
97XX1333	76	5544-LP30108-20	7.61	0.21	1.44	12.49	0.33	17	55.31	1.18	1.59	0.88	0.74
97XX1333	77	5544-LP30108-20	6.42	0.15	1.51	10.79	0.09	15.96	58.77	1.12	1.53	0.98	0.85
97XX1333	78	5544-LP30108-20	4.59	0.16	0.93	12.1	0.08	15.94	60.15	1.12	1.69	0.74	0.88
97XX1333	79	5544-LP30108-20	5.24	0.09	1.94	14.08	0.21	19.79	53.58	1.05	1.03	0.96	0.84

Table 11

CYCLE ID	SPL NO	STRAIN ID	16:0	16:1	18:0	18:1	18:2_Δ6,9	18:2_Δ9,12	18:3_Δ6,9, 12	18:3_Δ9,12, 15	18:4	20:0	20:1
97XX1333	80	5544-LP30108-20	4.38	0.08	1.66	22.25	0	30.79	35.49	2.16	0.72	0.66	0.84
97XX1333	81	5544-LP30108-20	4.05	0.05	1.44	24.16	0.04	24.86	40.89	1.42	0.79	0.63	0.84
97XX1333	82	5544-LP30108-20	3.29	0.05	1.9	19.66	0	23.83	46.48	1.27	0.87	0.78	0.81
97XX1333	83	5544-LP30108-20	4.82	0.08	1.99	17.27	0.1	20.69	49.73	1.22	1.06	0.98	0.82
97XX1333	84	5544-LP30108-20	5.33	0.1	1.77	13.6	0.03	21.44	51.74	1.52	1.21	0.98	0.93
97XX1333	85	5544-LP30108-20	3.3	0.05	1.2	68.23	0	22.09	0.01	2.27	0	0.57	1.57
97XX1333	86	5544-LP30108-20	3.23	0.05	1.54	28.15	0.01	36.4	25.91	1.99	0.43	0.59	0.97
97XX1333	87	5544-LP30108-20	4.38	0.1	1.16	60.94	2.85	8.35	17.61	1.26	0.69	0.54	1.39
97XX1333	88	5544-LP30108-20	4.4	0.09	1.34	38.42	0.02	34.74	16.61	2.12	0.32	0.53	0.82
97XX1278	16	5544-LP30108-15	3.62	0.11	1.22	27.23	0	30.9	32.87	1.41	0.48	0.46	0.97
97XX1278	17	5544-LP30108-15	3.68	0.13	1.26	45.29	0	44.79	0.72	1.77	0.01	0.43	1.24
97XX1278	18	5544-LP30108-15	4.08	0.15	1.49	22.34	0	28.37	39.37	1.22	0.64	0.55	0.88
97XX1278	19	5544-LP30108-15	3.51	0.1	1.01	35.44	0	44.12	11.7	1.72	0.15	0.36	1.14
97XX1278	20	5544-LP30108-15	3.66	0.12	1.21	27.44	0	30.2	32.37	1.49	0.53	0.49	1.15
97XX1278	21	5544-LP30108-15	3.58	0.11	1.51	29.81	0	30.72	30.65	1.16	0.4	0.5	0.96
97XX1278	23	5544-LP30108-15	3.69	0.11	1.42	30.05	0	32.28	27.41	1.65	0.38	0.54	1.19
97XX1278	24	5544-LP30108-15	3.56	0.11	1.31	30.25	0	28.64	31.46	1.43	0.48	0.48	1.11

Table 11

CYCLE ID	SPL NO	STRAIN ID	16:0	16:1	18:0	18:1	18:2_Δ6,9	18:2_Δ9,12	18:3_Δ6,9, 12	18:3_Δ9,12, 15	18:4	20:0	20:1
97XX1278	25	5544-LP30108-15	4.41	0.22	2.08	15.05	0	23.77	49.51	1.18	0.96	0.87	0.85
97XX1278	26	5544-LP30108-15	3.75	0.14	1.59	23.55	0	27.91	38.8	1.39	0.61	0.59	0.97
97XX1278	27	5544-LP30108-15	3.67	0.11	1.9	26.07	0	31.1	33.16	1.08	0.49	0.65	0.97
97XX1278	28	5544-LP30108-15	3.82	0.11	1.54	21.27	0	29.07	39.69	1.47	0.7	0.58	0.86
97XX1278	29	5544-LP30108-15	3.65	0.14	1.27	45.84	0	43.38	1	2.33	0.02	0.42	1.27
97XX1278	30	5544-LP30108-15	3.59	0.12	1.19	30.41	0	30.68	30.37	1.24	0.4	0.37	0.99
97XX1278	31	5544-LP30108-15	3.74	0.12	1.26	38.98	0	50.53	0.98	2.12	0.02	0.39	1.14
97XX1278	32	5544-LP30108-15	3.86	0.11	1.46	26.38	0	28.9	35.41	1.01	0.5	0.54	0.97

Example 11

Simultaneous expression of *M. alpina* $\Delta 5$ and $\Delta 6$ desaturases in *Brassica napus*

5 In order to produce arachadonic acid (ARA) in transgenic canola oil both $\Delta 5$ and $\Delta 6$ desaturase activities need to be introduced. In order to facilitate downstream characterization and breeding, it may be advantageous to have both activities encoded by a single T-DNA. The following example illustrates the simultaneous expression of $\Delta 5$ and $\Delta 6$ desaturases.

10 The Asp718 fragment of pCGN5528 containing the napin 5' regulatory region, the Ma29 coding region, and the napin 3' regulatory region was inserted into the Asp718 site of pCGN5138 to create pCGN5545. The NotI fragment of pCGN5536 containing the napin 5' regulatory region, the Ma524 coding region, and the napin 3' regulatory region was inserted into the NotI site of pCGN5545
15 to create pCGN5546. The expression modules were oriented in such a way that the direction of transcription from Ma524 and Ma29 and the nptII marker is the same.

 PCGN5546 was introduced into *Brassica napus* cv.LP30108 via
20 *Agrobacterium* mediated transformation. Mature selfed T2 seeds were collected from 30 independent LP30108 transformation events that were grown in the greenhouse. The fatty acid composition of 20-seed pools was analyzed by GC. The results are shown in Table 12. All the lines show expression of both desaturases as evidenced by the presence of $\Delta^{5,9}$ 18:2 (as seen in pCGN5531 plants) and $\Delta^{6,9}$ 18:2 and GLA (as seen in pCGN5538 plants)

25 .

Table 12

fatty acid analysis of 20-seed pools of mature T2 seeds from 5546-LP30108 events

STRAIN ID	16:0	16:1	18:0	18:1	18:2_Δ5,9	18:2_Δ6,9	18:2_Δ9,12	18:3_Δ6,9, 12	18:3_Δ9,12, 15	18:4	20:0	20:1
5546-LP30108-1	4.88	0.33	2.28	57.2	4.68	6.08	7.36	12.29	1.38	0.85	0.84	1.22
5546-LP30108-2	4.01	0.14	2.22	66.04	2.73	1.33	12.6	6.45	1.41	0.32	0.75	1.2
5546-LP30108-3	4.29	0.15	2.55	68.89	0.44	0.58	16.97	1.66	1.6	0.11	0.88	1.22
5546-LP30108-4	4.24	0.14	2.6	70.48	0.73	0.52	14.28	2.61	1.42	0.14	0.96	1.26
5546-LP30108-5	3.52	0.15	2.01	60.3	1.72	0.95	16.92	9.88	1.66	0.39	0.68	1.26
5546-LP30108-6	4.05	0.17	2.24	61.29	1.98	0.4	18.87	6.28	2	0.34	0.7	1.24
5546-LP30108-7	4.74	0.21	2.49	64.5	2.25	1.18	10.03	9.73	1.35	0.52	0.97	1.28
5546-LP30108-8	4.24	0.14	2.82	63.92	1.9	1.5	11.67	9.29	1.44	0.43	0.89	1.19
5546-LP30108-9	3.8	0.13	2.15	65.75	2.3	0.16	14.92	6.32	1.57	0.24	0.75	1.35
5546-LP30108-10	4.28	0.17	1.55	58.8	1.1	0.12	22.95	5.97	2.24	0.22	0.6	1.35
5546-LP30108-11	4.25	0.15	1.82	63.68	1.01	0.22	19.42	4.96	1.81	0.2	0.67	1.23
5546-LP30108-12	3.95	0.14	2.36	66.9	1.12	0.01	19.42	1.59	1.77	0.04	0.8	1.21
5546-LP30108-13	4.18	0.16	2.17	66.91	1.36	0.02	18.84	1.99	1.74	0.05	0.77	1.15
5546-LP30108-14	4.74	0.26	1.82	65.29	1.25	0.27	16.77	5.3	1.59	0.25	0.71	1.32
5546-LP30108-15	4.3	0.23	2.54	65.65	1.67	0.59	13.15	7.22	1.54	0.36	0.88	1.3
5546-LP30108-16	4.05	0.17	2.75	64.13	2.56	2.8	9.56	9.31	1.34	0.53	0.92	1.28

Table 12

fatty acid analysis of 20-seed pools of mature T2 seeds from 5546-LP30108 events

STRAIN ID	16:0	16:1	18:0	18:1	18:2_Δ5,9	18:2_Δ6,9	18:2_Δ9,12	18:3_Δ6,9, 12	18:3_Δ9,12, 15	18:4	20:0	20:1
5546-LP30108-17	4.06	0.13	2.85	65.76	2.09	1.92	9.65	9.1	1.23	0.45	0.92	1.22
5546-LP30108-18	4.16	0.25	2.14	60.68	1.43	0.02	24.02	2.62	2.11	0.09	0.69	1.26
5546-LP30108-19	5.77	0.37	2.15	56.11	1.6	0.33	19.34	9.16	2.37	0.46	0.73	1.05
5546-LP30108-20	5.03	0.36	2.34	61.05	1.55	0.35	17.21	6.96	2.24	0.39	0.77	1.22
5546-LP30108-21	4.52	0.3	2.71	62.14	1.33	0.23	17.62	6.44	1.88	0.28	0.88	1.15
5546-LP30108-22	5.91	0.44	2.15	60.12	1.41	0.36	17.04	7.75	1.97	0.36	0.78	1.07
5546-LP30108-23	4.28	0.22	2.44	66.19	0.93	0.11	17.03	4.37	1.67	0.17	0.82	1.25
5546-LP30108-24	4.92	0.33	2.68	62.6	1.32	0.36	16.89	5.82	2.05	0.3	0.95	1.19
5546-LP30108-25	5.42	0.72	3.15	47.47	2.66	4.21	13.51	16.31	2.14	0.99	1.18	1.37
5546-LP30108-26	3.85	0.22	2.78	65.02	1.05	0.05	18.35	4.36	1.67	0.12	0.82	1.18
5546-LP30108-27	3.86	0.15	2.76	65.17	1.11	0.78	16.24	5.21	1.53	0.25	0.93	1.3
5546-LP30108-28	5.29	0.42	1.81	49.12	1.07	0.09	30.52	5.21	3.57	0.44	0.67	1.23
5546-LP30108-29	4.4	0.2	2.38	65.95	1.05	0.28	16.31	4.85	1.64	0.19	0.85	1.26
5546-LP30108-30	3.99	0.19	2.55	67.47	0.83	0.11	17.02	3.18	1.68	0.13	0.83	1.23

Example 12

Simultaneous expression of *M. alpina* $\Delta 5$, $\Delta 6$ and $\Delta 12$ desaturases in *Brassica napus*

5 In order to achieve optimal production of ARA in transgenic canola oil both the $\Delta 6$ and $\Delta 12$ desaturase activities may need to be present in addition to the $\Delta 5$ activity. In order to facilitate downstream characterization and breeding, it may be advantageous to have all of these activities encoded by a single T-DNA. The following example illustrates the simultaneous expression of $\Delta 5$, $\Delta 6$ and $\Delta 12$ desaturases.

10 The HindIII fragment of pCGN5528 containing the napin 5' regulatory region, the Ma29 coding region, and the napin 3' regulatory region was inserted into the HindIII site of pCGN5544 to create pCGN5547. The expression modules were oriented in such a way that the direction of transcription from Ma29, Ma524, Ma648 and the nptII marker is the same.

15 PCGN5547 was introduced into *Brassica napus* cv.LP30108 via *Agrobacterium* mediated transformation. Mature selfed T2 seeds were collected from 30 independent LP30108 transformation events that were grown in the greenhouse. The fatty acid composition of 20-seed pools was analyzed by GC.

20 The results are shown in Table 13. Twenty-seven of the lines show significant accumulation of GLA and in general the levels of GLA observed are higher than those seen in the 5546 plants that did not contain the $\Delta 12$ desaturase. The $\Delta 12$ desaturase appears to be active in most lines as evidenced by the lack of detectable $\Delta 6,9$ 18:2 and elevated 18:2 levels in most plants. Small amounts of

25 $\Delta 5,9$ 18:2 are seen in the 5547 plants, although the levels are generally less than those observed in the 5546 plants. This may be due to the presence of the $\Delta 12$ desaturase which efficiently converts the 18:1 to 18:2 before it can be desaturated at the $\Delta 5$ position.

Table 13

fatty acid analysis of 20-seed pools of mature T2 seeds from 5547-LP30108 events

STRAIN ID	12:0	16:0	16:1	18:0	18:1	18:2_Δ5, 9	18:2_Δ6,9	18:2_Δ9,12	18:3_Δ6,9, 12	18:3_Δ9,12, 15	18:4	20:0	20:1	22:1	22:2
5547-LP30108-1	0.0	5.38	0.3	2.23	64.12	0.01	0	22.67	0.44	2.17	0.07	0.82	1.11	0.03	0
5547-LP30108-2	0.1	4.45	0.13	2.29	51.57	0.16	0	33.85	3.18	1.74	0.03	0.78	1.02	0.03	0.02
5547-LP30108-3	0.0	4.18	0.12	2.03	59.61	0.03	0	29.44	0.44	1.64	0	0.75	1.15	0.03	0.01
5547-LP30108-4	0.0	4.35	0.15	2.29	50.59	0.12	0.01	37.31	0.85	1.86	0.02	0.78	1.02	0.02	0.01
5547-LP30108-5	0.0	4.59	0.14	1.83	49	0.25	0.01	31.65	8.16	1.86	0.13	0.68	1.04	0.02	0
5547-LP30108-6	0.0	4.11	0.15	2.53	44.3	0.13	0	28.12	15.89	1.94	0.28	0.82	1.13	0	0
5547-LP30108-7	0.0	4.27	0.15	2.55	39.18	0.12	0.02	27	21.72	1.87	0.45	0.89	1.08	0	0
5547-LP30108-8	0.0	4.3	0.14	2.92	42.83	0.26	0	30.81	14.51	1.49	0.22	0.89	1.06	0	0
5547-LP30108-9	0.0	4.46	0.17	3.13	44.51	0	0	30.12	12.87	1.76	0.22	0.98	1.12	0.01	0
5547-LP30108-10	0.0	4.28	0.11	2.62	41.44	0.28	0	30.89	16.28	1.45	0.21	0.82	1.06	0	0
5547-LP30108-11	0.0	4.47	0.17	2.43	26.96	0.48	0	34.44	25.01	2.14	0.63	0.84	0.99	0	0
5547-LP30108-12	0.0	4.36	0.16	2.68	42.2	0.17	0	29.78	15.93	1.83	0.27	0.88	1.06	0	0
5547-LP30108-13	0.0	4.87	0.19	2.81	21.7	0.53	0	32.83	30.54	2.04	0.8	1	0.89	0.02	0.01
5547-LP30108-14	0.0	4.61	0.25	2.6	54	0	0	32.98	0.5	2.46	0.03	0.86	1.14	0	0
5547-LP30108-15	0.0	4.07	0.14	2.98	37.09	0.14	0.01	29.01	21.55	1.66	0.38	1.06	1.11	0	0

Table 13

fatty acid analysis of 20-seed pools of mature T2 seeds from 5547-LP30108 events

STRAIN ID	12:0	16:0	16:1	18:0	18:1	18:2_Δ5, 9	18:2_Δ6,9	18:2_Δ9,12	18:3_Δ6,9, 12	18:3_Δ9,12, 15	18:4	20:0	20:1	22:1	22:2	
5547-LP30108-16	0.0	3.63	0.13	2.12	64.69	0	0	0	24.21	0.15	2.04	0	0.82	1.56	0.02	0
5547-LP30108-17	0.0	3.85	0.18	2.22	67.22	0.01	0	0	21.25	0	2.27	0	0.83	1.53	0	0
5547-LP30108-18	0.0	5.46	0.19	2.87	41.83	0.1	0.04	0	22.76	21.45	1.72	0.48	1.06	1.23	0	0
5547-LP30108-19	0.0	4.33	0.12	2.73	50.31	0.07	0	0	24.77	12.72	1.62	0.21	1.04	1.29	0	0.01
5547-LP30108-20	0.0	4.22	0.12	2.91	46.33	0.25	0	0	26.87	14.65	1.61	0.22	0.98	1.18	0	0
5547-LP30108-21	0.0	4.38	0.17	2.37	55.37	0	0	0	32.59	0.53	1.85	0.03	0.83	1.23	0	0
5547-LP30108-22	0.0	5.5	0.18	2.71	41.93	0.1	0.19	0	24.19	20.14	1.76	0.45	0.94	1.21	0	0
5547-LP30108-23	0.0	4.03	0.16	2.17	68.44	0	0	0	20.09	0	2.19	0.02	0.83	1.46	0	0
5547-LP30108-24	0.0	4.19	0.17	2.72	49.31	0	0	0	30.38	8.64	1.85	0.13	0.86	1.16	0	0
5547-LP30108-25	0.0	4.04	0.17	2.1	70.48	0	0	0	18.04	0.05	2.09	0	0.86	1.54	0	0
5547-LP30108-26	0.0	4.74	0.22	3.2	26.74	0.33	0	0	30.05	28.95	2.02	0.78	1.08	0.99	0	0
5547-LP30108-27	0.0	4.29	0.18	2.23	52.49	0	0	0	28.48	7.36	1.91	0.13	0.87	1.37	0	0
5547-LP30108-28	0.0	4.36	0.17	3	44.35	0.2	0	0	29.59	13.39	1.91	0.23	0.96	1.17	0	0
5547-LP30108-29	0.0	4.32	0.17	2.94	52.53	0.05	0	0	33.88	0.91	2.34	0.01	0.97	1.23	0	0
5547-LP30108-30	0.0	4.07	0.14	2.89	45.13	0.01	0	0	29.06	13.96	1.71	0.2	0.94	1.2	0.01	0

Example 13

Stereospecific Distribution of $\Delta 6$ -Desaturated Oils

This experiment was designed to investigate the stereospecific distribution of the $\Delta 6$ -desaturated oils in seeds expressing pCGN5538 (Ma 524 cDNA). Three seed samples were used:

- 1) Non-transformed *B. napus* cv. LP004 seeds (control)
- 2) Segregating T2 seeds of pCGN5538-LP004-19
- 3) Segregating T2 seeds of pCGN5538-LP004-29

The following protocol was used for the analysis:

1. Seed Oil Extraction

Fifty seeds were placed in a 12 x 32 mm vial and crushed with a glass rod. 1.25 mL hexane was added and the mixture was vortexed. The seeds were extracted overnight on a shaker. The extract was then filtered through a 0.2 micron filter attached to a 1cc syringe. The extract was then dried down under nitrogen. The resulting oil was used for digestion and derivatization of the whole oil sample.

2. Digestion

A. Liquid Oil Digestion

The stock lipase (from *Rhizopus arrhizus*, Sigma, L4384) was diluted to approximately 600,000 units/mL with a goal of obtaining 50% digestion of the TAG. The stock lipase is maintained at 4 degrees C and placed on ice. The amount of reagents may be adjusted according to the amount of oil to be digested.

The following amounts are based on a 2.0 mg extracted oil sample. In a 12 x 32 mm screw cap vial the following were added: 2.0 mg oil, 200 μ L 0.1 M tris HCl pH 7, 40 μ L 2.2 w/v% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 100 μ L 0.05 w/v % bile salts. The material was vortexed and sonicated to disperse the oil. Twenty μ L of diluted lipase was added and the mixture was vortexed continuously for 1.0

minute at room temperature. A white precipitate formed. The reaction was stopped with 100 μ L 6M HCl and vortexing. Five hundred μ L CHCl_3 : CH_3OH (2:1) was added and the mixture was vortexed and held on ice while reaining digestions were carried out. Samples were vortexed again and centrifuged
5 briefly to sharpen layers. The lower layer containing digest products was removed with a pasteur pipette and placed in a 12 x 32 mm crimp cap vial. The material was then re-extracted with 300 μ L CHCl_3 , vortexed, centrifuged, and combined with the lower layers. The digest products were kept on ice as much as possible. HPLC separation is performed as soon as possible after digestion to
10 minimize acyl migration.

B. Solid Fat Digestion

The procedure for liquid oil digestion described above was followed except that 20 μ l 11:0 methyl ester is added to 2.0 mg solid fat.

3. HPLC Separation

15 The digestion products were dried down in chloroform to approximately 200 μ L. Each sample was then transferred into an insert in an 8 x 40 mm shell vial and 30 μ L was injected for HPLC analysis.

The high performance liquid chromatographic system was equipped with a Varex ELSD IIA evaporative light scattering detector with tube
20 temperature at 105°C and nitrogen gas flow at 40 mL/min; a Waters 712 Wisp autosampler, three Beckman 114M Solvent Delivery Modules; a Beckman 421A controller, a Rheodyne pneumatically actuated stream splitter; and a Gilson micro fractionator. The chromatography column is a 220 x 4.6 mm, 5 micron normal phase silica cartridge by Brownlee.

25 The three solvents used were:

A= hexane:toluene 1:1

B= toluene: ethyl acetate 3:1

C= 5% formic acid in ethyl acetate

The gradient profile was as follows:

Time (min)	Function	Value	Duration
0 flow	2.0 mL/min		
0 % B	10		
0 % C	2		
2 % C	25		6 min
14.0 % C	2		1 min
15.0	End program		

A chromatographic standard mixture is prepared in hexane:toluene 1:1 containing the following:

- 0.2 mg/mL triglyceride 16:0
- 5 2.0 mg/mL 16:0 Free Fatty Acid
- 0.2 mg/mL di16:0 mixed isomers (1,2-diacylglycerol and 1,3-diacylglycerol)
- 0.2 mg/mL 3-mono acylglycerol 16:0
- 0.2 mg/mL 2-mono acylglycerol 16:0

For each sample, the fraction containing the 2-mag peak is collected automatically by method controlled timed events relays. A time delay is used to synchronize the detector with the collector's emitter. The 2-mag peaks are collected and the fractions are evaporated at room temperature overnight.

The *sn*-2 composition results rely on minimization of acyl migration. Appearance of 1-monoacylglycerol and/or 3-monoacylglycerol peaks in the chromatograph means that acyl migration has occurred.

4. Derivatization

To derivatize the whole oil, 1.0 mg of the extracted whole oil was weighed into a 12 x 32 mm crimp cap vial. One mL toluene was then added. The sample is then vortexed and a 50 μ L aliquot was removed for derivatization. To the dried down 2-mag samples, 50 μ L toluene was added. To both the whole oil and 2-mag fractions 105 μ L H₂SO₄/CH₃OH @ 8.76 wt% is added. The cap was tightly capped and the sample is refluxed for 1 hour at 95 degrees C. The sample was allowed to cool and 500 μ L 10 w/v % NaCl in

water and 60 uL heptane was added. The organic layer was removed and inserted in a 12 x 32 mm crimp cap vial.

5. GLC Analysis

5 A Hewlett Packard model 6890 GC equipped with a split/splitless capillary inlet, FID detector, 6890 series autosampler and 3392A Alpha Omega integrator is set up for the capillary column as follows:

A. Supelco Omegawax 250, 30 m length, 0.25 mm id, 0.25 um film thickness

10	injection port:	260 C
	detector:	270 C
	initial temp:	170 C
	initial time:	1.5 min
	rate:	30 deg/min
15	final temp:	245 C
	final time:	6.5 min
	injection vol:	1.5 uL
	head pressure:	25 psi
	split ratio:	30
20	carrier gas:	He
	make-up gas:	N ₂
	FID gas:	H + air

Percent compositions of fatty acid methyl esters are calculated as mole percents. For carbon chain lengths less than 12, the use of theoretical or
25 empirical response factors in the area percent calculation is desirable.

6. Calculations

The mean distribution of each acyl group at each *sn*-1 and *sn*-3 position was calculated.

mean *sn*-1 and *sn*-3 composition = (3 WO comp - MAG comp) / 2

5 WO = whole oil

MAG= monoacylglycerol

The results of this analysis are presented in Table 14. The GLA and $\Delta^{6,9}$ 18:2 are evenly distributed between the *sn*-2 and *sn*-1, 3 positions. This analysis can not discriminate between fatty acids in the *sn*-1 vs. *sn*-3 positions.

Table 14

	16:0	16:1	18:0	18:1	18:2 Δ 6,9	18:2	18:3 Δ 6,9,12	8:3	18:4	20:0	20:1
Control											
sn2 composition	1.23	0.15	0.37	64.77	0.00	29.45	0.06	2.01	0.00	0.21	0.57
whole oil composition	4.33	0.20	3.32	69.29	0.18	18.51	0.00	1.35	0.06	0.91	1.17
mean sn1, sn3 composition*	5.88	0.23	4.80	71.55	0.27	13.04	-0.03	1.02	0.09	1.26	1.47
5538-19											
sn2 composition	1.65	0.27	4.12	57.21	5.61	14.55	12.45	1.38	0.32	0.43	1.00
whole oil composition	5.44	0.33	4.09	57.51	4.53	10.57	13.16	1.03	0.50	1.07	1.07
mean sn1, sn3 composition*	7.34	0.36	4.08	57.66	3.99	8.58	13.52	0.86	0.59	1.39	1.11
5538-29											
sn2 composition	1.24	0.27	1.56	56.35	6.35	17.85	12.99	1.60	0.38	0.14	0.40
whole oil composition	4.96	0.32	3.73	54.92	4.99	12.11	13.66	1.10	0.50	0.99	1.11
mean sn1, sn3 composition*	6.82	0.35	4.82	54.21	4.31	9.24	14.00	0.85	0.56	1.42	1.47
*calculated from the mag and whole oil composition for each analyte											

Example 14**Fatty Acid Compositions of Transgenic Plants**

$\Delta 5$ and $\Delta 6$ transgenic plants were analyzed for their fatty acid content.

The following protocol was used for oil extraction:

- 5 1. About 400 mg of seed were weighed out in duplicate for each sample.
2. The seeds were crushed in a mortar and pestle. The mortar and pestle was rinsed twice with 3ml (2:1) (v:v) CHCl_3 : CH_3OH /MeOH. An additional 6 ml (2:1) was added to
10 the 20ml glass vial (oil extracted in 12ml total 2:1).
3. Samples were vortexed and placed on an orbital shaker for 2 hours with occasional vortexing.
4. 5ml of 1M NaCl was added to each sample. Sample was vortexed then spun in centrifuge at 2000rpm for 5 minutes.
15 Lower phase was drawn off using a pasteur pipette.
5. Upper phase was re-extracted with an additional 5ml. Sample was vortexed then spun in centrifuge at 2000 rpm for 5 minutes. The lower phase was drawn off using a pasteur pipette and added to previous lower phase.
- 20 6. CHCl_3 : CH_3OH /MeOH was evaporated under nitrogen using evaporative cooling. Vial containing extracted oil was sealed under nitrogen. Between 120mg- 160mg oil was extracted for each sample.

25 For GC-MS analysis, fatty acid methyl esters were dissolved in an appropriate volume of hexane and analyzed using a Hewlett-Packard 5890 Series II Plus gas chromatograph (Hewlett Packard, Palo Alto, CA) equipped with a 30 m x 0.32 mm i.d. Omegawax 320 fused silica capillary column (Supelco, Bellefonte, PA) and a Hewlett-Packard 5972 Series mass selective detector. Mass spectra were interpreted by comparison to the mass spectra in

NIST/EPA/NIH Chemical Structure Database using a MS Chem Station
(#G1036A) (Hewlett Packard).

Transgenic line 5531-6 was analyzed in duplicate (A, B) and compared
to control line LP004-6. The fatty acid profile results are shown in Table 15.

- 5 Transgenic line 5538-19 was analyzed in duplicate (A, B) and compared
to control line LP004-6. The fatty acid profile results are shown in Table 16.

Table 15
Fatty Acid Profile

	CONTROL	CONTROL	TRANSGENIC	TRANSGENIC
	LP004-6A	LP004-6B	5531-6A	5531-6B
	LRL-2043	LRL-2044	LRL-2042	LRL-2045
	001f0102.d	001f0103.d	001f0101.d	001f0104.d
C12:0				
C13:0				
C14:0		0.053		0.061
C14:1				
C15:0 isomer				
C15:0				
C16:0	4.107	4.034	4.257	4.224
C16:1	0.181	0.173	0.200	0.199
C16:2	0.061	0.065	0.081	0.060
C17:0				
C16:3	0.244	0.246	0.155	0.151
C16:4				
C18:0	2.608	2.714	3.368	3.417
C18:1w9	65.489	66.454	59.529	59.073
C18:1w7	2.297	2.185	2.388	2.393
C18:2 5,9			6.144	6.269
C18:2w6	19.828	18.667	18.872	19.059
C18:3 5,9,12			0.469	0.496
C18:3w6		0.060		
C18:3w3	1.587	1.578	1.428	1.418
C18:4w6				
C18:4w3				
C20:0	0.962	0.998	1.009	1.022
C20:1w11	1.336	1.335	1.058	1.065
C20:1w9				
C20:1w7			0.076	0.080
C20:2w6	0.073	0.073		0.052
C20:3w6				

Table 15
Fatty Acid Profile

	CONTROL	CONTROL	TRANSGENIC	TRANSGENIC
	LP004-6A	LP004-6B	5531-6A	5531-6B
	LRL-2043	LRL-2044	LRL-2042	LRL-2045
	001f0102.d	001f0103.d	001f0101.d	001f0104.d
C20:4w6				
C20:3w3				
C20:4w3				
C20:5w3				
C22:0(1.000)	0.542	0.558	0.463	0.467
C22:1w11		0.038		
C22:1w9				
C22:1w7		0.034		
C21:5				
C23:0		0.029		
C22:4w6				
C22:5w6				
C22:5w3				
C24:0	0.373	0.391	0.280	0.283
C22:6w3	0.314	0.317	0.223	0.212
C24:1w9				
TOTAL	100.00	100.00	100.00	100.00

Table 16
Fatty Acid Profile

	5538-19A	5538-19B	LP004-6A	LP004-6B
	TRANSGENIC	TRANSGENIC	CONTROL	CONTROL
	LRL-2166	LRL-2167	LRL-2168	LRL-2169
C6:0	0.004	0.005		
C8:0	0.007	0.007	0.004	0.005
C10:0	0.012	0.012	0.008	0.008
C12:0	0.020	0.020	0.011	0.012
C13:0				
C14:0	0.099	0.108	0.050	0.050
C14:1w5				
C15:0	0.059	0.068	0.017	0.019
C16:0	5.272	5.294	4.049	4.057
C16:1	0.350	0.417	0.197	0.208
C16:2	0.199	0.187	0.076	0.077
C17:0	0.092	0.089	0.078	0.077
C16:3	0.149	0.149	0.192	0.198
C16:4		0.010		
C18:0	3.815	3.771	2.585	2.638
C18:1	57.562	57.051	68.506	68.352
C18:2 (6,9)	4.246	4.022		
C18:2w6	10.900	11.589	19.098	19.122
C18:2w3	0.020	0.008	0.008	0.009
C18:3w6	12.565	12.595	0.013	0.015
C18:3w3	1.084	1.137	1.501	1.542
C18:4	0.017	0.013	0.011	0.008
C18:4	0.028	0.024		
C20:0	1.138	1.104	0.937	0.943
C20:1	1.115	1.085	1.330	1.327
C20:2w6	0.150	0.143	0.068	0.071
C20:3w6	0.026	0.025	0.014	0.012
C20:4w6				
C20:3w3				

Table 16
Fatty Acid Profile

	5538-19A	5538-19B	LP004-6A	LP004-6B
	TRANSGENIC	TRANSGENIC	CONTROL	CONTROL
	LRL-2166	LRL-2167	LRL-2168	LRL-2169
C20:4w3				
C20:5w3				
C22:0	0.506	0.484	0.535	0.539
C22:1	0.017	0.020	0.032	0.032
C21:5		0.040	0.030	0.031
C22:4w6	0.038	0.064	0.015	0.014
C22:5w6				
C22:5w3	0.023	0.018	0.021	0.017
C24:0	0.352	0.321	0.353	0.362
C22:6w3	0.009			
C24:1w9	0.129	0.121	0.260	0.255
TOTAL	100.00	100.00	100.00	100.00

Example 15

Combined Expression of $\Delta 6$ and $\Delta 12$ Desaturases in *B. napus* Achieved by Crossing

Plants containing either the $\Delta 6$ or the $\Delta 12$ desaturase were crossed and individual F1 half-seeds were analyzed for fatty acid composition by GC. Data from one such cross are given in Table 17. The parents for the cross were 5538-LP004-25-2-25 ($\Delta 6$ expressor) and 5542-SP30021-10-16 ($\Delta 12$ expressor). Reciprocal crosses were made and the results of 25 individual F1 seeds of each are shown in the table. Crosses are described such that the first parent indicated is the female. Both sets of crosses gave approximately the same results. Compared to the parents, the $\Delta^{6,9}$ 18:2 decreased, and the GLA increased. $\Delta^{9,12}$ 18:2 levels are increased in most of the F1's as well. Note that these are F1 seeds and only contain one set of each desaturase. In future generations and selection of events homozygous for each desaturase, the F2 GLA levels obtained may be even higher.

Combining traits by crossing may be preferable to combining traits on one T-DNA in some situations. Particularly if both genes are driven off of the same promoter (in this case napin), issues of promoter silencing may favor this approach over putting multiple cDNAs on one construct.

Alternatively, in some cases, combining multiple cDNAs on one T-DNA may be the method of choice. The results are shown in Table 17.

Table 17

STRAIN ID	16:0	16:1	18:0	18:1	18:2_Δ6,9	18:2_Δ9,12	18:3_Δ6,9, 12	18:3_Δ9,12, 11	18:4	20:0	20:1
5538-LP004-25-2-25	4.23	0.13	2.4	61.78	8.77	6.34	11.58	0.92	0	0	0
5542-SP30021-10-16	4.09	0.1	2.03	38.4	0	41.88	0	11.06	0.02	0.75	1.03
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.9	0.04	2.31	38.58	0	27.91	20.94	2.67	0.65	0.92	1.28
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.5	0.04	1.88	36.24	0	28.68	22.54	3.36	0.85	0.78	1.32
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.51	0.03	1.98	38.36	0	29.48	19.95	3.06	0.68	0.79	1.38
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.95	0.04	1.86	38.65	0	28.08	20.81	2.92	0.75	0.76	1.42
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	4.26	0.05	2.44	40.25	0.01	28.81	18.08	2.74	0.53	0.88	1.24
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	4.13	0.04	2.33	34.48	0	26.73	26.2	2.32	0.75	0.9	1.27
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.8	0.04	2.15	38.34	0	28.95	20.64	2.63	0.65	0.81	1.3
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.96	0.05	1.59	36.43	0	29.05	21.85	3.47	0.86	0.68	1.32
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	4.04	0.04	2.5	37.75	0	27.23	22.89	1.95	0.55	0.99	1.26
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.53	0.04	1.8	34.88	0	29.17	23.42	3.42	0.9	0.74	1.3
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.43	0.04	1.89	37.12	0	29.52	20.91	3.35	0.8	0.79	1.35
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.58	0.03	2.55	39.54	0	28.81	19.34	2.44	0.54	0.98	1.34
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.53	0.03	2.33	39.26	0	29.07	19.5	2.61	0.59	0.91	1.37
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.4	0.02	2.41	45.53	0	28.94	13.71	2.51	0.37	0.91	1.44

Table 17

STRAIN ID	16:0	16:1	18:0	18:1	18:2_Δ6,9	18:2_Δ9,12	18:3_Δ6,9, 12	18:3_Δ9,12, 11	18:4	20:0	20:1
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.49	0.03	2.57	40.95	0	28.52	17.97	2.63	0.58	0.99	1.43
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.65	0.04	2.11	38.02	0	29.13	20.53	2.85	0.66	0.86	1.33
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.97	0.03	1.99	34.95	0.01	27.15	25.71	2.38	0.79	0.81	1.38
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.81	0.05	1.46	38.3	0	31.51	17.67	3.83	0.75	0.61	1.33
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.98	0.05	2.03	37.14	0	30.09	20.28	2.79	0.72	0.8	1.36
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	4.03	0.04	2.52	42.9	0	27.79	16.66	2.64	0.54	0.9	1.29
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	4.03	0.04	2.27	40.72	0	29.37	17.56	2.53	0.53	0.86	1.35
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.98	0.04	2.61	39.91	0	28.06	19.15	2.69	0.6	0.96	1.26
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	3.73	0.03	1.89	40.22	0	29.44	18.21	3	0.67	0.73	1.39
(5538-LP004-25-2-25 X 5542-SP30021-10-16)	4.02	0.04	2.14	42.58	0	30.36	15.18	2.43	0.42	0.82	1.3
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4.14	0.06	2.23	30.67	0	30.38	25.47	3.12	0.91	0.9	1.29
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4.05	0.07	1.7	37.03	0.04	32.1	15.97	5.38	0.96	0.69	1.28
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4.01	0.07	1.58	38.02	0.05	33.65	13.92	5.15	0.89	0.66	1.28
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4.07	0.06	2.01	31.63	0.05	31.13	23.09	3.94	1.1	0.83	1.28
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4.03	0.05	1.94	31.88	0	30.98	23.71	3.45	0.99	0.82	1.3
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	3.92	0.06	1.71	35.77	0.03	33.15	16.39	5.28	0.98	0.68	1.32
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4.09	0.08	1.57	34.6	0.03	33.73	16.73	5.48	0.99	0.66	1.28

Table 17

STRAIN ID	16:0	16:1	18:0	18:1	18:2_Δ6,9	18:2_Δ9,12	18:3_Δ6,9, 12	18:3_Δ9,12, 11	18:4	20:0	20:1
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	3.94	0.07	1.59	34.03	0.04	31.35	19.76	5.29	1.22	0.67	1.28
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4.13	0.06	1.85	31.44	0.06	31.28	23.77	3.52	1.04	0.79	1.22
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4.14	0.06	1.96	31.11	0.04	31.88	23.3	3.6	1.01	0.82	1.27
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	3.98	0.07	1.58	35.06	0	32.06	18.1	5.33	1.12	0.67	1.28
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	3.89	0.06	1.59	32.51	0.05	29.44	22.91	5.33	1.54	0.67	1.25
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4	0.07	1.69	32.1	0.05	30.49	22.77	4.66	1.32	0.75	1.26
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4.06	0.05	1.93	30.77	0.07	28.37	27.21	3.37	1.19	0.84	1.25
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4.1	0.06	1.9	31.77	0.05	32.33	22.03	3.92	0.98	0.78	1.27
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	3.94	0.07	1.67	34.74	0.03	33.63	17.1	5.16	0.99	0.68	1.26
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	3.71	0.06	1.65	33.05	0	33.22	19.73	4.7	1.07	0.68	1.39
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	3.84	0.06	1.71	34.16	0.04	34.52	16.74	5.18	0.97	0.68	1.34
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4	0.07	1.66	34.97	0.07	33.08	17.07	5.27	1.1	0.67	1.28
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4.16	0.06	1.99	35.44	0.05	31.89	18.95	3.68	0.89	0.81	1.29
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4.05	0.08	1.46	33.49	0	31.96	18.81	6.2	1.32	0.61	1.28
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4.2	0.06	1.93	35.06	0.06	33.69	17.38	4	0.86	0.78	1.21
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4.07	0.06	1.74	36	0.06	32.18	17.86	4.32	0.96	0.73	1.27
(5542-SP30021-10-16 X 5538-LP004-25-2-25)	4.11	0.05	2.24	29.64	0.04	28.64	27.94	3.06	1.12	0.97	1.26

Example 16

Expression of *M. alpina* desaturases in soybean

The *M. alpina* desaturases can be used to drive production of GLA and other PUFAs in soybean by use of the following expression constructs. Two means by which exogenous DNA can be inserted into the soybean genome are *Agrobacterium* infection or particle gun. Particle gun transformation is disclosed in U.S. patent 5,503,998. Plants can be selected using a glyphosate resistance marker (4, 971, 908). *Agrobacterium* transformation of soybean is well established to one of ordinary skill in the art.

For seed specific expression, the coding regions of the desaturase cDNAs are placed under control of the 5' regulatory region of *Glycine max* alpha-type beta conglycinin storage protein gene. The specific region that can be used is nucleotides 78-921 of gi 169928 (Doyle, J.J., Schuler, M.A., Godette, W.D., Zenger, V., Beachy, R.N., and Slightom, J.L., 1986 J. Biol. Chem. 261 (20), 9228-9238). The 3' regulatory region that can be used is from the pea ribulose 1,5 biphosphate carboxylase/oxygenase small subunit (rbcS) gene. The specific sequences to be used are nucleotides 1-645 of gi 169145 (Hunt, A.G. 1988 DNA 7: 329-336).

Since soybean seeds contain more 18:2, and perhaps more endogenous $\Delta 12$ desaturase activity than *Brassica*, the effect of the *Mortierella* $\Delta 12$ desaturase on achieving optimal GLA levels can be tested as follows. A construct containing the $\Delta 6$ cDNA can be used to see if $\Delta^{6,9}$ 18:2 is produced along with GLA. A construct containing the $\Delta 12$ desaturase can be used to see if the amount of 18:2 can be increased in soybean. A construct containing both the $\Delta 6$ and $\Delta 12$ desaturases can be used to produce optimal levels of GLA. Alternatively, plants containing each of the single desaturases may be crossed if necessary to combine the genes.

Similar constructs may be made to express the $\Delta 5$ desaturase alone, or in combination with $\Delta 12$ and/or $\Delta 6$ desaturases.

Example 17

Human Desaturase Gene Sequences

Human desaturase gene sequences potentially involved in long chain polyunsaturated fatty acid biosynthesis were isolated based on homology
5 between the human cDNA sequences and *Mortierella alpina* desaturase gene sequences. The three conserved "histidine boxes" known to be conserved among membrane-bound desaturases were found. As with some other membrane-bound desaturases the final HXXHH histidine box motif was found to be QXXHH. The amino acid sequence of the putative human desaturases
10 exhibited homology to *M. alpina* $\Delta 5$, $\Delta 6$, $\Delta 9$, and $\Delta 12$ desaturases.

The *M. alpina* $\Delta 5$ desaturase and $\Delta 6$ desaturase cDNA sequences were used to search the LifeSeq database of Incyte Pharmaceuticals, Inc., Palo Alto, California 94304. The $\Delta 5$ desaturase sequence was divided into fragments; 1) amino acid no. 1-150, 2) amino acid no. 151-300, and 3) amino acid no. 301-
15 446. The $\Delta 6$ desaturase sequence was divided into three fragments; 1) amino acid no. 1-150, 2) amino acid no. 151-300, and 3) amino acid no. 301-457. These polypeptide fragments were searched against the database using the "tblastn" algorithm. This algorithm compares a protein query sequence against a nucleotide sequence database dynamically translated in all six reading frames
20 (both strands).

The polypeptide fragments 2 and 3 of *M. alpina* $\Delta 5$ and $\Delta 6$ have homologies with the CloneID sequences as outlined in Table 18. The CloneID represents an individual sequence from the Incyte LifeSeq database. After the "tblastn" results have been reviewed, Clone Information was searched with the
25 default settings of Stringency of ≥ 50 , and Productscore ≤ 100 for different CloneID numbers. The Clone Information Results displayed the information including the ClusterID, CloneID, Library, HitID, Hit Description. When selected, the ClusterID number displayed the clone information of all the clones that belong in that ClusterID. The Assemble command assembles all of the
30 CloneID which comprise the ClusterID. The following default settings were

used for GCG (Genetics Computer Group, University of Wisconsin Biotechnology Center, Madison, Wisconsin 53705) Assembly:

	Word Size:	7
5	Minimum Overlap:	14
	Stringency:	0.8
	Minimum Identity:	14
	Maximum Gap:	10
	Gap Weight:	8
10	Length Weight:	2

GCG Assembly Results displayed the contigs generated on the basis of sequence information within the CloneID. A contig is an alignment of DNA sequences based on areas of homology among these sequences. A new
15 sequence (consensus sequence) was generated based on the aligned DNA sequences within a contig. The contig containing the CloneID was identified, and the ambiguous sites of the consensus sequence was edited based on the alignment of the CloneIDs (see SEQ ID NO:31 - SEQ ID NO:35) to generate the best possible sequence. The procedure was repeated for all six CloneID
20 listed in Table 18. This produced five unique contigs. The edited consensus sequences of the 5 contigs were imported into the Sequencher software program (Gene Codes Corporation, Ann Arbor, Michigan 48105). These consensus sequences were assembled. The contig 2511785 overlaps with contig 3506132, and this new contig was called 2535 (SEQ ID NO:37). The contigs from the
25 Sequencher program were copied into the Sequence Analysis software package of GCG.

Each contig was translated in all six reading frames into protein sequences. The *M. alpina* $\Delta 5$ (MA29) and $\Delta 6$ (MA524) sequences were compared with each of the translated contigs using the FastA search (a Pearson

and Lipman search for similarity between a query sequence and a group of sequences of the same type (nucleic acid or protein)). Homology among these sequences suggest the open reading frames of each contig. The homology among the *M. alpina* $\Delta 5$ and $\Delta 6$ to contigs 2535 and 3854933 were utilized to create the final contig called 253538a. Figure 9 is the FastA match of the final contig 253538a and MA29, and Figure 10 is the FastA match of the final contig 253538a and MA524. The DNA sequences for the various contigs are presented in SEQ ID NO:31 -SEQ ID NO:37 The various peptide sequences are shown in SEQ ID NO:38 - SEQ ID NO: 44.

Although the open reading frame was generated by merging the two contigs, the contig 2535 shows that there is a unique sequence in the beginning of this contig which does not match with the contig 3854933. Therefore, it is possible that these contigs were generated from independent desaturase like human genes.

The contig 253538a contains an open reading frame encoding 432 amino acids. It starts with Gln (CAG) and ends with the stop codon (TGA). The contig 253538a aligns with both *M. alpina* $\Delta 5$ and $\Delta 6$ sequences, suggesting that it could be either of the desaturases, as well as other known desaturases which share homology with each other. The individual contigs listed in Table 18, as well as the intermediate contig 2535 and the final contig 253538a can be utilized to isolate the complete genes for human desaturases.

Uses of the Human Desaturases

These human sequences can be expressed in yeast and plants utilizing the procedures described in the preceding examples. For expression in mammalian cells and transgenic animals, these genes may provide superior codon bias. In addition, these sequences can be used to isolate related desaturase genes from other organisms.

Table 18

Sections of the Desaturases	Clone ID from LifeSeq Database	Keyword
151-300 $\Delta 5$	3808675	fatty acid desaturase
301-446 $\Delta 5$	354535	$\Delta 6$
151-300 $\Delta 6$	3448789	$\Delta 6$
151-300 $\Delta 6$	1362863	$\Delta 6$
151-300 $\Delta 6$	2394760	$\Delta 6$
301-457 $\Delta 6$	3350263	$\Delta 6$

Example 18**5 Identification of Homologues to *M. alpina* $\Delta 5$ and $\Delta 6$ desaturases**

A nucleic acid sequence that encodes a putative $\Delta 5$ desaturase was identified through a TBLASTN search of the expressed sequence tag databases through NCBI using amino acids 100-446 of Ma29 as a query. The truncated portion of the Ma29 sequence was used to avoid picking up homologies based on the cytochrome b5 portion at the N-terminus of the desaturase. The deduced amino acid sequence of an est from *Dictyostelium discoideum* (accession # C25549) shows very significant homology to Ma29 and lesser, but still significant homology to Ma524. The DNA sequence is presented as SEQ ID NO:45. The amino acid sequence is presented as SEQ ID NO:46.

15 Example 19**Identification of *M. alpina* $\Delta 5$ and $\Delta 6$ homologues in other PUFA-producing organisms**

To look for desaturases involved in PUFA production, a cDNA library was constructed from total RNA isolated from *Phaeodactylum tricornutum*. A plasmid-based cDNA library was constructed in pSPORT1 (GIBCO-BRL) following manufacturer's instructions using a commercially available kit (GIBCO-BRL). Random cDNA clones were sequenced and nucleic acid sequences that encode putative $\Delta 5$ or $\Delta 6$ desaturases were identified through BLAST search of the databases and comparison to Ma29 and Ma524 sequences.

One clone was identified from the *Phaeodactylum* library with homology to Ma29 and Ma524; it is called 144-011-B12. The DNA sequence is presented as SEQ ID NO:47. The amino acid sequence is presented as SEQ ID NO:48.

5

Example 20

Identification of *M. alpina* $\Delta 5$ and $\Delta 6$ homologues in other PUFA-producing organisms

To look for desaturases involved in PUFA production, a cDNA library was constructed from total RNA isolated from *Schizochytrium* species. A plasmid-based cDNA library was constructed in pSPORT1 (GIBCO-BRL) following manufacturer's instructions using a commercially available kit (GIBCO-BRL). Random cDNA clones were sequenced and nucleic acid sequences that encode putative $\Delta 5$ or $\Delta 6$ desaturases were identified through BLAST search of the databases and comparison to Ma29 and Ma524 sequences.

15

One clone was identified from the *Schizochytrium* library with homology to Ma29 and Ma524; it is called 81-23-C7. This clone contains a ~1 kb insert. Partial sequence was obtained from each end of the clone using the universal forward and reverse sequencing primers. The DNA sequence from the forward primer is presented as SEQ ID NO:49. The peptide sequence is presented as SEQ ID NO:50. The DNA sequence from the reverse primer is presented as SEQ ID NO:51. The amino acid sequence from the reverse primer is presented as SEQ ID NO:52.

20

Example 21

Nutritional Compositions

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The PUFAs of the previous examples can be utilized in various nutritional supplements, infant formulations, nutritional substitutes and other nutrition solutions.

I. INFANT FORMULATIONS

A. Isomil® Soy Formula with Iron.

Usage: As a beverage for infants, children and adults with an allergy or sensitivity to cow's milk. A feeding for patients with disorders for which lactose should be avoided: lactase deficiency, lactose intolerance and galactosemia.

5

Features:

- Soy protein isolate to avoid symptoms of cow's-milk-protein allergy or sensitivity
- Lactose-free formulation to avoid lactose-associated diarrhea
- Low osmolality (240 mOsm/kg water) to reduce risk of osmotic diarrhea.

10

- Dual carbohydrates (corn syrup and sucrose) designed to enhance carbohydrate absorption and reduce the risk of exceeding the absorptive capacity of the damaged gut.

15

- 1.8 mg of Iron (as ferrous sulfate) per 100 Calories to help prevent iron deficiency.
- Recommended levels of vitamins and minerals.
- Vegetable oils to provide recommended levels of essential fatty acids.
- Milk-white color, milk-like consistency and pleasant aroma.

20

Ingredients: (Pareve, ©) 85% water, 4.9% corn syrup, 2.6% sugar (sucrose), 2.1% soy oil, 1.9% soy protein isolate, 1.4% coconut oil, 0.15% calcium citrate, 0.11 % calcium phosphate tribasic, potassium citrate, potassium phosphate monobasic, potassium chloride, mono- and diglycerides, soy lecithin, carrageenan, ascorbic acid, L-methionine, magnesium chloride, potassium phosphate dibasic, sodium chloride, choline chloride, taurine, ferrous sulfate, m-inositol, alpha-tocopheryl acetate, zinc sulfate, L-carnitine, niacinamide, calcium pantothenate, cupric sulfate, vitamin A palmitate, thiamine chloride hydrochloride, riboflavin, pyridoxine hydrochloride, folic

25

acid, manganese sulfate, potassium iodide, phylloquinone, biotin, sodium selenite, vitamin D₃ and cyanocobalamin.

B. Isomil® DF Soy Formula For Diarrhea.

5 Usage: As a short-term feeding for the dietary management of diarrhea in infants and toddlers.

Features:

- First infant formula to contain added dietary fiber from soy fiber specifically for diarrhea management.
- 10 • Clinically shown to reduce the duration of loose, watery stools during mild to severe diarrhea in infants.
- Nutritionally complete to meet the nutritional needs of the infant.
- Soy protein isolate with added L-methionine meets or exceeds an infant's requirement for all essential amino acids.
- Lactose-free formulation to avoid lactose-associated diarrhea.
- 15 • Low osmolality (240 mOsm/kg water) to reduce the risk of osmotic diarrhea.
- Dual carbohydrates (corn syrup and sucrose) designed to enhance carbohydrate absorption and reduce the risk of exceeding the absorptive capacity of the damaged gut.
- 20 • Meets or exceeds the vitamin and mineral levels recommended by the Committee on Nutrition of the American Academy of Pediatrics and required by the Infant Formula Act.
- 1.8 mg of iron (as ferrous sulfate) per 100 Calories to help prevent iron deficiency.
- 25 • Vegetable oils to provide recommended levels of essential fatty acids.

Ingredients: (Pareve, ®) 86% water, 4.8% corn syrup, 2.5% sugar (sucrose), 2.1% soy oil, 2.0% soy protein isolate, 1.4% coconut oil, 0.77% soy

fiber, 0.12% calcium citrate, 0.11 % calcium phosphate tribasic, 0.10% potassium citrate, potassium chloride, potassium phosphate monobasic, mono- and diglycerides, soy lecithin, carrageenan, magnesium chloride, ascorbic acid, L-methionine, potassium phosphate dibasic, sodium chloride, choline chloride, 5 taurine, ferrous sulfate, m-inositol, alpha-tocopheryl acetate, zinc sulfate, L-carnitine, niacinamide, calcium pantothenate, cupric sulfate, vitamin A palmitate, thiamine chloride hydrochloride, riboflavin, pyridoxine hydrochloride, folic acid, manganese sulfate, potassium iodide, phylloquinone, biotin, sodium selenite, vitamin D₃ and cyanocobalamin.

10 **C. Isomil® SF Sucrose-Free Soy Formula With Iron.**

Usage: As a beverage for infants, children and adults with an allergy or sensitivity to cow's-milk protein or an intolerance to sucrose. A feeding for patients with disorders for which lactose and sucrose should be avoided.

Features:

- 15 • Soy protein isolate to avoid symptoms of cow's-milk-protein allergy or sensitivity.
- Lactose-free formulation to avoid lactose-associated diarrhea (carbohydrate source is Polycose® Glucose Polymers).
- Sucrose free for the patient who cannot tolerate sucrose.
- 20 • Low osmolality (180 mOsm/kg water) to reduce risk of osmotic diarrhea.
- 1.8 mg of iron (as ferrous sulfate) per 100 Calories to help prevent iron deficiency.
- Recommended levels of vitamins and minerals.
- 25 • Vegetable oils to provide recommended levels of essential fatty acids.
- Milk-white color, milk-like consistency and pleasant aroma.

Ingredients: (Pareve, ®) 75% water, 11.8% hydrolyzed cornstarch, 4.1% soy oil, 4.1% soy protein isolate, 2.8% coconut oil, 1.0% modified cornstarch,

0.38% calcium phosphate tribasic, 0.17% potassium citrate, 0.13% potassium chloride, mono- and diglycerides, soy lecithin, magnesium chloride, ascorbic acid, L-methionine, calcium carbonate, sodium chloride, choline chloride, carrageenan, taurine, ferrous sulfate, m-inositol, alpha-tocopheryl acetate, zinc sulfate, L-carnitine, niacinamide, calcium pantothenate, cupric sulfate, vitamin A palmitate, thiamine chloride hydrochloride, riboflavin, pyridoxine hydrochloride, folic acid, manganese sulfate, potassium iodide, phylloquinone, biotin, sodium selenite, vitamin D₃ and cyanocobalamin.

**D. Isomil® 20 Soy Formula With Iron Ready To Feed,
20 Cal/fl oz.**

Usage: When a soy feeding is desired.

Ingredients: (Pareve, ®) 85% water, 4.9% corn syrup, 2.6% sugar (sucrose), 2.1% soy oil, 1.9% soy protein isolate, 1.4% coconut oil, 0.15% calcium citrate, 0.11% calcium phosphate tribasic, potassium citrate, potassium phosphate monobasic, potassium chloride, mono- and diglycerides, soy lecithin, carrageenan, ascorbic acid, L-methionine, magnesium chloride, potassium phosphate dibasic, sodium chloride, choline chloride, taurine, ferrous sulfate, m-inositol, alpha-tocopheryl acetate, zinc sulfate, L-carnitine, niacinamide, calcium pantothenate, cupric sulfate, vitamin A palmitate, thiamine chloride hydrochloride, riboflavin, pyridoxine hydrochloride, folic acid, manganese sulfate, potassium iodide, phylloquinone, biotin, sodium selenite, vitamin D₃ and cyanocobalamin.

E. Similac® Infant Formula

Usage: When an infant formula is needed: if the decision is made to discontinue breastfeeding before age 1 year, if a supplement to breastfeeding is needed or as a routine feeding if breastfeeding is not adopted.

Features:

- Protein of appropriate quality and quantity for good growth; heat-denatured, which reduces the risk of milk-associated enteric blood loss.
- 5 • Fat from a blend of vegetable oils (doubly homogenized), providing essential linoleic acid that is easily absorbed.
- Carbohydrate as lactose in proportion similar to that of human milk.
- Low renal solute load to minimize stress on developing organs.
- 10 • Powder, Concentrated Liquid and Ready To Feed forms.

Ingredients: (®-D) Water, nonfat milk, lactose, soy oil, coconut oil, mono- and diglycerides, soy lecithin, ascorbic acid, carrageenan, choline chloride, taurine, m-inositol, alpha-tocopheryl acetate, zinc sulfate, niacinamid, ferrous sulfate, calcium pantothenate, cupric sulfate, vitamin A palmitate, 15 thiamine chloride hydrochloride, riboflavin, pyridoxine hydrochloride, folic acid, manganese sulfate, phylloquinone, biotin, sodium selenite, vitamin D₃ and cyanocobalamin.

F. Similac® NeoCare Premature Infant Formula With Iron

Usage: For premature infants' special nutritional needs after hospital 20 discharge. Similac NeoCare is a nutritionally complete formula developed to provide premature infants with extra calories, protein, vitamins and minerals needed to promote catch-up growth and support development.

Features:

- 25 • Reduces the need for caloric and vitamin supplementation. More calories (22 Cal/fl oz) than standard term formulas (20 Cal/fl oz).
- Highly absorbed fat blend, with medium-chain triglycerides (MCT oil) to help meet the special digestive needs of premature infants.
- Higher levels of protein, vitamins and minerals per 100 Calories to extend the nutritional support initiated in-hospital.

- More calcium and phosphorus for improved bone mineralization.

Ingredients: ©-D Corn syrup solids, nonfat milk, lactose, whey protein concentrate, soy oil, high-oleic safflower oil, fractionated coconut oil (medium-chain triglycerides), coconut oil, potassium citrate, calcium phosphate tribasic, calcium carbonate, ascorbic acid, magnesium chloride, potassium chloride, sodium chloride, taurine, ferrous sulfate, m-inositol, choline chloride, ascorbyl palmitate, L-carnitine, alpha-tocopheryl acetate, zinc sulfate, niacinamide, mixed tocopherols, sodium citrate, calcium pantothenate, cupric sulfate, thiamine chloride hydrochloride, vitamin A palmitate, beta carotene, riboflavin, pyridoxine hydrochloride, folic acid, manganese sulfate, phylloquinone, biotin, sodium selenite, vitamin D₃ and cyanocobalamin.

G. Similac Natural Care Low-Iron Human Milk Fortifier Ready To Use, 24 Cal/fl oz.

Usage: Designed to be mixed with human milk or to be fed alternatively with human milk to low-birth-weight infants.

Ingredients: ©-D Water, nonfat milk, hydrolyzed cornstarch, lactose, fractionated coconut oil (medium-chain triglycerides), whey protein concentrate, soy oil, coconut oil, calcium phosphate tribasic, potassium citrate, magnesium chloride, sodium citrate, ascorbic acid, calcium carbonate, mono- and diglycerides, soy lecithin, carrageenan, choline chloride, m-inositol, taurine, niacinamide, L-carnitine, alpha tocopheryl acetate, zinc sulfate, potassium chloride, calcium pantothenate, ferrous sulfate, cupric sulfate, riboflavin, vitamin A palmitate, thiamine chloride hydrochloride, pyridoxine hydrochloride, biotin, folic acid, manganese sulfate, phylloquinone, vitamin D₃, sodium selenite and cyanocobalamin.

Various PUFAs of this invention can be substituted and/or added to the infant formulae described above and to other infant formulae known to those in the art..

II. NUTRITIONAL FORMULATIONS

A. ENSURE®

Usage: ENSURE is a low-residue liquid food designed primarily as an oral nutritional supplement to be used with or between meals or, in appropriate amounts, as a meal replacement. ENSURE is lactose- and gluten-free, and is suitable for use in modified diets, including low-cholesterol diets. Although it is primarily an oral supplement, it can be fed by tube.

Patient Conditions:

- For patients on modified diets
- For elderly patients at nutrition risk
- For patients with involuntary weight loss
- For patients recovering from illness or surgery
- For patients who need a low-residue diet

Ingredients:

®-D Water, Sugar (Sucrose), Maltodextrin (Corn), Calcium and Sodium Caseinates, High-Oleic Safflower Oil, Soy Protein Isolate, Soy Oil, Canola Oil, Potassium Citrate, Calcium Phosphate Tribasic, Sodium Citrate, Magnesium Chloride, Magnesium Phosphate Dibasic, Artificial Flavor, Sodium Chloride, Soy Lecithin, Choline Chloride, Ascorbic Acid, Carrageenan, Zinc Sulfate, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Gellan Gum, Niacinamide, Calcium Pantothenate, Manganese Sulfate, Cupric Sulfate, Vitamin A Palmitate, Thiamine Chloride Hydrochloride, Pyridoxine Hydrochloride, Riboflavin, Folic Acid, Sodium Molybdate, Chromium Chloride, Biotin, Potassium Iodide, Sodium Selenate.

B. ENSURE® BARS

Usage: ENSURE BARS are complete, balanced nutrition for supplemental use between or with meals. They provide a delicious, nutrient-

rich alternative to other snacks. ENSURE BARS contain <1 g lactose/bar, and Chocolate Fudge Brownie flavor is gluten-free. (Honey Graham Crunch flavor contains gluten.)

Patient Conditions:

- 5
- For patients who need extra calories, protein, vitamins and minerals
 - Especially useful for people who do not take in enough calories and nutrients
 - For people who have the ability to chew and swallow
 - Not to be used by anyone with a peanut allergy or any type of allergy to
- 10 nuts.

Ingredients:

- Honey Graham Crunch -- High-Fructose Corn Syrup, Soy Protein-Isolate, Brown Sugar, Honey, Maltodextrin (Corn), Crisp Rice (Milled Rice, Sugar [Sucrose], Salt [Sodium Chloride] and Malt), Oat Bran, Partially
- 15 Hydrogenated Cottonseed and Soy Oils, Soy Polysaccharide, Glycerine, Whey Protein Concentrate, Polydextrose, Fructose, Calcium Caseinate, Cocoa Powder, Artificial Flavors, Canola Oil, High-Oleic Safflower Oil, Nonfat Dry Milk, Whey Powder, Soy Lecithin and Corn Oil. Manufactured in a facility that processes nuts.

20 **Vitamins and Minerals:**

- Calcium Phosphate Tribasic, Potassium Phosphate Dibasic, Magnesium Oxide, Salt (Sodium Chloride), Potassium Chloride, Ascorbic Acid, Ferric Orthophosphate, Alpha-Tocopheryl Acetate, Niacinamide, Zinc Oxide, Calcium Pantothenate, Copper Gluconate, Manganese Sulfate, Riboflavin, Beta-
- 25 Carotene, Pyridoxine Hydrochloride, Thiamine Mononitrate, Folic Acid, Biotin, Chromium Chloride, Potassium Iodide, Sodium Selenate, Sodium Molybdate, Phylloquinone, Vitamin D₃ and Cyanocobalamin.

Protein:

Honey Graham Crunch - The protein source is a blend of soy protein isolate and milk proteins.

	Soy protein isolate	74%
5	Milk proteins	26%

Fat:

Honey Graham Crunch - The fat source is a blend of partially hydrogenated cottonseed and soybean, canola, high oleic safflower, and corn oils, and soy lecithin.

10	Partially hydrogenated cottonseed and soybean oil	76%
	Canola oil	8%
	High-oleic safflower oil	8%
	Corn oil	4%
	Soy lecithin	4%

15 Carbohydrate:

Honey Graham Crunch - The carbohydrate source is a combination of high-fructose corn syrup, brown sugar, maltodextrin, honey, crisp rice, glycerine, soy polysaccharide, and oat bran.

	High-fructose corn syrup	24%
20	Brown sugar	21%
	Maltodextrin	12%
	Honey	11%
	Crisp rice	9%
	Glycerine	9%
25	Soy polysaccharide	7%
	Oat bran	7%\

C. ENSURE® HIGH PROTEIN

Usage: ENSURE HIGH PROTEIN is a concentrated, high-protein liquid food designed for people who require additional calories, protein, vitamins, and minerals in their diets. It can be used as an oral nutritional supplement with or between meals or, in appropriate amounts, as a meal replacement. ENSURE HIGH PROTEIN is lactose- and gluten-free, and is suitable for use by people recovering from general surgery or hip fractures and by patients at risk for pressure ulcers.

Patient Conditions

- For patients who require additional calories, protein, vitamins, and minerals, such as patients recovering from general surgery or hip fractures, patients at risk for pressure ulcers, and patients on low-cholesterol diets

Features-

- Low in saturated fat
- Contains 6 g of total fat and < 5 mg of cholesterol per serving
- Rich, creamy taste
- Excellent source of protein, calcium, and other essential vitamins and minerals
- For low-cholesterol diets
- Lactose-free, easily digested

Ingredients:

Vanilla Supreme: -D Water, Sugar (Sucrose), Maltodextrin (Corn), Calcium and Sodium Caseinates, High-Oleic Safflower Oil, Soy Protein Isolate, Soy Oil, Canola Oil, Potassium Citrate, Calcium Phosphate Tribasic, Sodium Citrate, Magnesium Chloride, Magnesium Phosphate Dibasic, Artificial Flavor, Sodium Chloride, Soy Lecithin, Choline Chloride, Ascorbic Acid, Carrageenan, Zinc Sulfate, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Gellan Gum, Niacinamide, Calcium Pantothenate, Manganese Sulfate, Cupric Sulfate, Vitamin A Palmitate, Thiamine Chloride Hydrochloride, Pyridoxine Hydrochloride,

Riboflavin, Folio Acid, Sodium Motybdate, Chromium Chloride, Biotin, Potassium Iodide, Sodium Selenate, Phylloquinone, Vitamin D.3 and Cyanocobalarnin.

Protein:

- 5 The protein source is a blend of two high-biologic-value proteins: casein and soy.

Sodium and calcium caseinates	85%
Soy protein isolate	15%

Fat:

- 10 The fat source is a blend of three oils: high-oleic safflower, canola, and soy.

High-oleic safflower oil	40%
Canola oil	30%
Soy oil	30%

- 15 The level of fat in ENSURE HIGH PROTEIN meets American Heart Association (AHA) guidelines. The 6 grams of fat in ENSURE HIGH PROTEIN represent 24% of the total calories, with 2.6% of the fat being from saturated fatty acids and 7.9% from polyunsaturated fatty acids. These values are within the AHA guidelines of $\leq 30\%$ of total calories from fat, $< 1\ 0\%$ of the calories from saturated fatty acids, and $\leq 1\ 0\%$ of total calories from
- 20 polyunsaturated fatty acids.

Carbohydrate:

- 25 ENSURE HIGH PROTEIN contains a combination of maltodextrin and sucrose. The mild sweetness and flavor variety (vanilla supreme, chocolate royal, wild berry, and banana), plus VARI-FLAVORSO® Flavor Pacs in pecan, cherry, strawberry, lemon, and orange, help to prevent flavor fatigue and aid in patient compliance.

Vanilla and other nonchocolate flavors

Sucrose	60%
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Maltodextrin	40%
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Chocolate

Sucrose	70%
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Maltodextrin	30%
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5

D. ENSURE® LIGHT

Usage: ENSURE LIGHT is a low-fat liquid food designed for use as an oral nutritional supplement with or between meals. ENSURE LIGHT is lactose- and gluten-free, and is suitable for use in modified diets, including low-cholesterol diets.

10

Patient Conditions:

- For normal-weight or overweight patients who need extra nutrition in a supplement that contains 50% less fat and 20% fewer calories than ENSURE
- For healthy adults who don't eat right and need extra nutrition

15

Features:

- Low in fat and saturated fat
- Contains 3 g of total fat per serving and < 5 mg cholesterol
- Rich, creamy taste
- Excellent source of calcium and other essential vitamins and minerals
- For low-cholesterol diets
- Lactose-free, easily digested

20

Ingredients:

French Vanilla: ®-D Water, Maltodextrin (Corn), Sugar (Sucrose), Calcium Caseinate, High-Oleic Safflower Oil, Canola Oil, Magnesium Chloride, Sodium Citrate, Potassium Citrate, Potassium Phosphate Dibasic, Magnesium Phosphate Dibasic, Natural and Artificial Flavor, Calcium Phosphate Tribasic, Cellulose Gel, Choline Chloride, Soy Lecithin, Carrageenan, Salt (Sodium Chloride),

25

- Ascorbic Acid, Cellulose Gum, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Zinc Sulfate, Niacinamide, Manganese Sulfate, Calcium Pantothenate, Cupric Sulfate, Thiamine Chloride Hydrochloride, Vitamin A Palmitate, Pyridoxine Hydrochloride, Riboflavin, Chromium Chloride, Folic Acid, Sodium
- 5 Molybdate, Biotin, Potassium Iodide, Sodium Selenate, Phylloquinone, Vitamin D₃ and Cyanocobalamin.

Protein:

The protein source is calcium caseinate.

Calcium caseinate	100%
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10 **Fat**

The fat source is a blend of two oils: high-oleic safflower and canola.

High-oleic safflower oil	70%
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Canola oil	30%
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- 15 The level of fat in ENSURE LIGHT meets American Heart Association (AHA) guidelines. The 3 grams of fat in ENSURE LIGHT represent 13.5% of the total calories, with 1.4% of the fat being from saturated fatty acids and 2.6% from polyunsaturated fatty acids. These values are within the AHA guidelines of $\leq 30\%$ of total calories from fat, $< 10\%$ of the calories from saturated fatty acids, and $\leq 10\%$ of total calories from polyunsaturated fatty acids.

20 **Carbohydrate**

ENSURE LIGHT contains a combination of maltodextrin and sucrose. The chocolate flavor contains corn syrup as well. The mild sweetness and flavor variety (French vanilla, chocolate supreme, strawberry swirl), plus VARI-FLAVORS® Flavor Pacs in pecan, cherry, strawberry, lemon, and

25 orange, help to prevent flavor fatigue and aid in patient compliance.

Vanilla and other nonchocolate flavors

Sucrose	51%
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Maltodextrin	49%
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Chocolate

Sucrose	47.0%
Corn Syrup	26.5%
Maltodextrin	26.5%

5 **Vitamins and Minerals**

An 8-fl-oz serving of ENSURE LIGHT provides at least 25% of the RDIs for 24 key vitamins and minerals.

Caffeine

Chocolate flavor contains 2.1 mg caffeine/8 fl oz.

10

E. ENSURE PLUS®

Usage: ENSURE PLUS is a high-calorie, low-residue liquid food for use when extra calories and nutrients, but a normal concentration of protein, are needed. It is designed primarily as an oral nutritional supplement to be used with or between meals or, in appropriate amounts, as a meal replacement. ENSURE PLUS is lactose- and gluten-free. Although it is primarily an oral nutritional supplement, it can be fed by tube.

15

Patient Conditions:

- For patients who require extra calories and nutrients, but a normal concentration of protein, in a limited volume
- For patients who need to gain or maintain healthy weight

20

Features

- Rich, creamy taste
- Good source of essential vitamins and minerals

25

Ingredients

Vanilla: ®-D Water, Corn Syrup, Maltodextrin (Corn), Corn Oil, Sodium and Calcium Caseinates, Sugar (Sucrose), Soy Protein Isolate, Magnesium Chloride,

- Potassium Citrate, Calcium Phosphate Tribasic, Soy Lecithin, Natural and Artificial Flavor, Sodium Citrate, Potassium Chloride, Choline Chloride, Ascorbic Acid, Carrageenan, Zinc Sulfate, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Niacinamide, Calcium Pantothenate, Manganese Sulfate, Cupric Sulfate, Thiamine Chloride Hydrochloride, Pyridoxine Hydrochloride, Riboflavin, Vitamin A Palmitate, Folic Acid, Biotin, Chromium Chloride, Sodium Molybdate, Potassium Iodide, Sodium Selenite, Phylloquinone, Cyanocobalamin and Vitamin D₃.

Protein

- 10 The protein source is a blend of two high-biologic-value proteins: casein and soy.

Sodium and calcium caseinates	84%
Soy protein isolate	16%

Fat

- 15 The fat source is corn oil.

Corn oil	100%
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Carbohydrate

- 20 ENSURE PLUS contains a combination of maltodextrin and sucrose. The mild sweetness and flavor variety (vanilla, chocolate, strawberry, coffee, butter pecan, and eggnog), plus VARI-FLAVORS® Flavor Pacs in pecan, cherry, strawberry, lemon, and orange, help to prevent flavor fatigue and aid in patient compliance.

Vanilla, strawberry, butter pecan, and coffee flavors

25	Corn Syrup	39%
	Maltodextrin	38%
	Sucrose	23%

Chocolate and eggnog flavors

Corn Syrup	36%
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Maltodextrin	34%
Sucrose	30%

Vitamins and Minerals

5 An 8-fl-oz serving of ENSURE PLUS provides at least 15% of the RDIs
for 25 key Vitamins and minerals.

Caffeine

Chocolate flavor contains 3.1 mg Caffeine/8 fl oz. Coffee flavor
contains a trace amount of caffeine.

10 **F. ENSURE PLUS® HN**

Usage: ENSURE PLUS HN is a nutritionally complete high-calorie,
high-nitrogen liquid food designed for people with higher calorie and protein
needs or limited volume tolerance. It may be used for oral supplementation or
for total nutritional support by tube. ENSURE PLUS HN is lactose- and gluten-
15 free.

Patient Conditions:

- For patients with increased calorie and protein needs, such as following surgery or injury
- For patients with limited volume tolerance and early satiety

20 **Features**

- For supplemental or total nutrition
- For oral or tube feeding
- 1.5 CaV/mL
- High nitrogen
- 25 • Calorically dense

Ingredients

Vanilla: ©-D Water, Maltodextrin (Corn), Sodium and Calcium Caseinates, Corn Oil, Sugar (Sucrose), Soy Protein Isolate, Magnesium Chloride, Potassium Citrate, Calcium Phosphate Tribasic, Soy Lecithin, Natural and Artificial
5 Flavor, Sodium Citrate, Choline Chloride, Ascorbic Acid, Taurine, L-Carnitine, Zinc Sulfate, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Niacinamide, Carrageenan, Calcium Pantothenate, Manganese Sulfate, Cupric Sulfate, Thiamine Chloride Hydrochloride, Pyridoxine Hydrochloride, Riboflavin, Vitamin A Palmitate, Folic Acid, Biotin, Chromium Chloride, Sodium
10 Molybdate, Potassium Iodide, Sodium Selenite, Phylloquinone, Cyanocobalamin and Vitamin D₃.

G. ENSURE® POWDER

Usage: ENSURE POWDER (reconstituted with water) is a low-residue
15 liquid food designed primarily as an oral nutritional supplement to be used with or between meals. ENSURE POWDER is lactose- and gluten-free, and is suitable for use in modified diets, including low-cholesterol diets.

Patient Conditions:

- For patients on modified diets
- 20 • For elderly patients at nutrition risk
- For patients recovering from illness/surgery
- For patients who need a low-residue diet

Features

- Convenient, easy to mix
- 25 • Low in saturated fat
- Contains 9 g of total fat and < 5 mg of cholesterol per serving
- High in vitamins and minerals
- For low-cholesterol diets

- Lactose-free, easily digested

Ingredients: ©-D Corn Syrup, Maltodextrin (Corn), Sugar (Sucrose), Corn Oil, Sodium and Calcium Caseinates, Soy Protein Isolate, Artificial Flavor, Potassium Citrate, Magnesium Chloride, Sodium Citrate, Calcium Phosphate

5 Tribasic, Potassium Chloride, Soy Lecithin, Ascorbic Acid, Choline Chloride, Zinc Sulfate, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Niacinamide, Calcium Pantothenate, Manganese Sulfate, Thiamine Chloride Hydrochloride, Cupric Sulfate, Pyridoxine Hydrochloride, Riboflavin, Vitamin A Palmitate, Folic Acid, Biotin, Sodium Molybdate, Chromium Chloride, Potassium Iodide,

10 Sodium Selenate, Phylloquinone, Vitamin D₃ and Cyanocobalamin.

Protein

The protein source is a blend of two high-biologic-value proteins: casein and soy.

	Sodium and calcium caseinates	84%
15	Soy protein isolate	16%

Fat

The fat source is corn oil.

Corn oil	100%
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Carbohydrate

20 ENSURE POWDER contains a combination of corn syrup, maltodextrin, and sucrose. The mild sweetness of ENSURE POWDER, plus VARI-FLAVORS® Flavor Pacs in pecan, cherry, strawberry, lemon, and orange, helps to prevent flavor fatigue and aid in patient compliance.

Vanilla

25	Corn Syrup	35%
	Maltodextrin	35%
	Sucrose	30%

H. ENSURE® PUDDING

Usage: ENSURE PUDDING is a nutrient-dense supplement providing balanced nutrition in a nonliquid form to be used with or between meals. It is appropriate for consistency-modified diets (e.g., soft, pureed, or full liquid) or for people with swallowing impairments. ENSURE PUDDING is gluten-free.

Patient Conditions:

- For patients on consistency-modified diets (e.g., soft, pureed, or full liquid)
- For patients with swallowing impairments

Features

- Rich and creamy, good taste
- Good source of essential vitamins and minerals Convenient-needs no refrigeration
- Gluten-free

Nutrient Profile per 5 oz: Calories 250, Protein 10.9%, Total Fat 34.9%, Carbohydrate 54.2%

Ingredients:

Vanilla: ©-D Nonfat Milk, Water, Sugar (Sucrose), Partially Hydrogenated Soybean Oil, Modified Food Starch, Magnesium Sulfate, Sodium Stearoyl Lactylate, Sodium Phosphate Dibasic, Artificial Flavor, Ascorbic Acid, Zinc Sulfate, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Choline Chloride, Niacinamide, Manganese Sulfate, Calcium Pantothenate, FD&C Yellow #5, Potassium Citrate, Cupric Sulfate, Vitamin A Palmitate, Thiamine Chloride Hydrochloride, Pyridoxine Hydrochloride, Riboflavin, FD&C Yellow #6, Folic Acid, Biotin, Phylloquinone, Vitamin D3 and Cyanocobalamin.

Protein

The protein source is nonfat milk.

Nonfat milk

100%

Fat

The fat source is hydrogenated soybean oil.

Hydrogenated soybean oil	100%
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Carbohydrate

5 ENSURE PUDDING contains a combination of sucrose and modified food starch. The mild sweetness and flavor variety (vanilla, chocolate, butterscotch, and tapioca) help prevent flavor fatigue. The product contains 9.2 grams of lactose per serving.

Vanilla and other nonchocolate flavors

10	Sucrose	56%
	Lactose	27%
	Modified food starch	17%

Chocolate

	Sucrose	58%
15	Lactose	26%
	Modified food starch	16%

I. ENSURE® WITH FIBER

20 Usage: ENSURE WITH FIBER is a fiber-containing, nutritionally complete liquid food designed for people who can benefit from increased dietary fiber and nutrients. ENSURE WITH FIBER is suitable for people who do not require a low-residue diet. It can be fed orally or by tube, and can be used as a nutritional supplement to a regular diet or, in appropriate amounts, as a meal replacement. ENSURE WITH FIBER is lactose- and gluten-free, and is

25 suitable for use in modified diets, including low-cholesterol diets.

Patient Conditions

- For patients who can benefit from increased dietary fiber and nutrients

Features

- New advanced formula-low in saturated fat, higher in vitamins and minerals
- Contains 6 g of total fat and < 5 mg of cholesterol per serving
- Rich, creamy taste
- 5 • Good source of fiber
- Excellent source of essential vitamins and minerals
- For low-cholesterol diets
- Lactose- and gluten-free

Ingredients

- 10 **Vanilla:** ®-D Water, Maltodextrin (Corn), Sugar (Sucrose), Sodium and Calcium Caseinates, Oat Fiber, High-Oleic Safflower Oil, Canola Oil, Soy Protein Isolate, Corn Oil, Soy Fiber, Calcium Phosphate Tribasic, Magnesium Chloride, Potassium Citrate, Cellulose Gel, Soy Lecithin, Potassium Phosphate Dibasic, Sodium Citrate, Natural and Artificial Flavors, Choline Chloride,
- 15 Magnesium Phosphate, Ascorbic Acid, Cellulose Gum, Potassium Chloride, Carrageenan, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Zinc Sulfate, Niacinamide, Manganese Sulfate, Calcium Pantothenate, Cupric Sulfate, Vitamin A Palmitate, Thiamine Chloride Hydrochloride, Pyridoxine Hydrochloride, Riboflavin, Folic Acid, Chromium Chloride, Biotin, Sodium
- 20 Molybdate, Potassium Iodide, Sodium Selenate, Phylloquinone, Vitamin D₃ and Cyanocobalamin.

Protein

The protein source is a blend of two high-biologic-value proteins- casein and soy.

25	Sodium and calcium caseinates	80%
	Soy protein isolate	20%

Fat

The fat source is a blend of three oils: high-oleic safflower, canola, and corn.

	High-oleic safflower oil	40%
5	Canola oil	40%
	Corn oil	20%

The level of fat in ENSURE WITH FIBER meets American Heart Association (AHA) guidelines. The 6 grams of fat in ENSURE WITH FIBER represent 22% of the total calories, with 2.01 % of the fat being from saturated fatty acids and 6.7% from polyunsaturated fatty acids. These values are within the AHA guidelines of $\leq 30\%$ of total calories from fat, $< 10\%$ of the calories from saturated fatty acids, and $\leq 10\%$ of total calories from polyunsaturated fatty acids.

Carbohydrate

ENSURE WITH FIBER contains a combination of maltodextrin and sucrose. The mild sweetness and flavor variety (vanilla, chocolate, and butter pecan), plus VARI-FLAVORS® Flavor Pacs in pecan, cherry, strawberry, lemon, and orange, help to prevent flavor fatigue and aid in patient compliance.

Vanilla and other nonchocolate flavors

20	Maltodextrin	66%
	Sucrose	25%
	Oat Fiber	7%
	Soy Fiber	2%

Chocolate

25	Maltodextrin	55%
	Sucrose	36%
	Oat Fiber	7%

Soy Fiber

2%

Fiber

The fiber blend used in ENSURE WITH FIBER consists of oat fiber and soy polysaccharide. This blend results in approximately 4 grams of total dietary fiber per 8-fl-oz can. The ratio of insoluble to soluble fiber is 95:5.

The various nutritional supplements described above and known to others of skill in the art can be substituted and/or supplemented with the PUFAs of this invention.

J. Oxepa™ Nutritional Product

Oxepa is low-carbohydrate, calorically dense enteral nutritional product designed for the dietary management of patients with or at risk for ARDS. It has a unique combination of ingredients, including a patented oil blend containing eicosapentaenoic acid (EPA from fish oil), γ -linolenic acid (GLA from borage oil), and elevated antioxidant levels.

Caloric Distribution:

- Caloric density is high at 1.5 Cal/mL (355 Cal/8 fl oz), to minimize the volume required to meet energy needs.
- The distribution of Calories in Oxepa is shown in Table 7.

Table 7. Caloric Distribution of Oxepa			
	per 8 fl oz.	per liter	% of Cal
Calories	355	1,500	---
Fat (g)	22.2	93.7	55.2
Carbohydrate (g)	25	105.5	28.1
Protein (g)	14.8	62.5	16.7
Water (g)	186	785	---

Fat:

- Oxepa contains 22.2 g of fat per 8-fl oz serving (93.7 g/L).
- The fat source is a oil blend of 31.8% canola oil, 25% medium-chain triglycerides (MCTs), 20% borage oil, 20% fish oil, and 3.2 % soy lecithin. The typical fatty acid profile of Oxepa is shown in Table 8.

- Oxepa provides a balanced amount of polyunsaturated, monounsaturated, and saturated fatty acids, as shown in Table 10.
- Medium-chain triglycerides (MCTs) -- 25% of the fat blend -- aid gastric emptying because they are absorbed by the intestinal tract without emulsification by bile acids.

5

The various fatty acid components of Oxepa™ nutritional product can be substituted and/or supplemented with the PUFAs of this invention.

Table 8. Typical Fatty Acid Profile

	% Total Fatty Acids	g/8 fl oz*	g/L*
Caproic (6:0)	0.2	0.04	0.18
Caprylic (8:0)	14.69	3.1	13.07
Capric (10:0)	11.06	2.33	9.87
Palmitic (16:0)	5.59	1.18	4.98
Palmitoleic (16:1n-7)	1.82	0.38	1.62
Stearic (18:0)	1.84	0.39	1.64
Oleic (18:1n-9)	24.44	5.16	21.75
Linoleic (18:2n-6)	16.28	3.44	14.49
α -Linolenic (18:3n-3)	3.47	0.73	3.09
γ -Linolenic (18:3n-6)	4.82	1.02	4.29
Eicosapentaenoic (20:5n-3)	5.11	1.08	4.55
n-3-Docosapentaenoic (22:5n-3)	0.55	0.12	0.49
Docosahexaenoic (22:6n-3)	2.27	0.48	2.02
Others	7.55	1.52	6.72

* Fatty acids equal approximately 95% of total fat.

Table 9. Fat Profile of Oxepa.

% of total calories from fat	55.2
Polyunsaturated fatty acids	31.44 g/L
Monounsaturated fatty acids	25.53 g/L
Saturated fatty acids	32.38 g/L
n-6 to n-3 ratio	1.75:1
Cholesterol	9.49 mg/8 fl oz 40.1 mg/L

Carbohydrate:

- The carbohydrate content is 25.0 g per 8-fl-oz serving (105.5 g/L).
- The carbohydrate sources are 45% maltodextrin (a complex carbohydrate) and 55% sucrose (a simple sugar), both of which are readily digested and absorbed.

5

- The high-fat and low-carbohydrate content of Oxepa is designed to minimize carbon dioxide (CO₂) production. High CO₂ levels can complicate weaning in ventilator-dependent patients. The low level of carbohydrate also may be useful for those patients who have developed stress-induced hyperglycemia.

10

- Oxepa is lactose-free.

Dietary carbohydrate, the amino acids from protein, and the glycerol moiety of fats can be converted to glucose within the body. Throughout this process, the carbohydrate requirements of glucose-dependent tissues (such as the central nervous system and red blood cells) are met. However, a diet free of carbohydrates can lead to ketosis, excessive catabolism of tissue protein, and loss of fluid and electrolytes. These effects can be prevented by daily ingestion of 50 to 100 g of digestible carbohydrate, if caloric intake is adequate. The carbohydrate level in Oxepa is also sufficient to minimize gluconeogenesis, if energy needs are being met.

15

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Protein:

- Oxepa contains 14.8 g of protein per 8-fl-oz serving (62.5 g/L).
- The total calorie/nitrogen ratio (150:1) meets the need of stressed patients.
- Oxepa provides enough protein to promote anabolism and the maintenance of lean body mass without precipitating respiratory problems. High protein intakes are a concern in patients with respiratory insufficiency. Although protein has little effect on CO₂ production, a high protein diet will increase ventilatory drive.

25

- The protein sources of Oxepa are 86.8% sodium caseinate and 13.2% calcium caseinate.
- As demonstrated in Table 11, the amino acid profile of the protein system in Oxepa meets or surpasses the standard for high quality protein set by the National Academy of Sciences.
- Oxepa is gluten-free.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

SEQUENCE LISTING

5

(1) GENERAL INFORMATION:

- 10 (i) APPLICANT: KNUTZON, DEBORAH
MURKERJI, PRADIP
HUANG, YUNG-SHENG
THURMOND, JENNIFER
CHAUDHARY, SUNITA
LEONARD, AMANDA
- 15 (ii) TITLE OF INVENTION: METHODS AND COMPOSITIONS FOR SYNTHESIS
OF LONG CHAIN POLY-UNSATURATED FATTY ACIDS IN PLANTS
- (iii) NUMBER OF SEQUENCES: 52
- 20 (iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: LIMBACH & LIMBACH L.L.P.
(B) STREET: 2001 FERRY BUILDING
(C) CITY: SAN FRANCISCO
(D) STATE: CA
25 (E) COUNTRY: USA
(F) ZIP: 94111
- (v) COMPUTER READABLE FORM:
30 (A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: Microsoft Word
- 35 (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:
- 40 (vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/834,033
(B) FILING DATE: 11-APR-1997
- (vii) PRIOR APPLICATION DATA:
45 (A) APPLICATION NUMBER: US 08/833,610
(B) FILING DATE: 11-APR-1997
- (viii) ATTORNEY/AGENT INFORMATION:
50 (A) NAME: MICHAEL R. WARD
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(C) REFERENCE/DOCKET NUMBER: CGAB-320
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(C) TELEX: N/A

(2) INFORMATION FOR SEQ ID NO:1:

60

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1617 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: DNA (genomic)

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

	CGACACTCCT TCCTTCTTCT CACCCGTCCT AGTCCCCTTC AACCCCCCTC TTTGACAAAG	60
	ACAACAAACC ATGGCTGCTG CTCCCAGTGT GAGGACGTTT ACTCGGGCCG AGGTTTTGAA	120
15	TGCCGAGGCT CTGAATGAGG GCAAGAAGGA TGCCGAGGCA CCCTTCTTGA TGATCATCGA	180
	CAACAAGGTG TACGATGTCC GCGAGTTCGT CCCTGATCAT CCCGGTGGAA GTGTGATTCT	240
20	CACGCACGTT GGCAAGGACG GCACTGACGT CTTTGACACT TTTACCCCCG AGGCTGCTTG	300
	GGAGACTCTT GCCAACTTTT ACGTTGGTGA TATTGACGAG AGCGACCGCG ATATCAAGAA	360
	TGATGACTTT GCGGCCGAGG TCCGCAAGCT GCGTACCTTG TTCCAGTCTC TTGGTTACTA	420
25	CGATTCTTCC AAGGCATACT ACGCCTTCAA GGTCTCGTTC AACCTCTGCA TCTGGGGTTT	480
	GTCGACGGTC ATTGTGGCCA AGTGGGGCCA GACCTCGACC CTCGCCAACG TGCTCTCGGC	540
30	TGCGCTTTTG GGTCTGTTCT GGCAGCAGTG CGGATGGTTG GCTCACGACT TTTTGCATCA	600
	CCAGGTCTTC CAGGACCGTT TCTGGGGTGA TCTTTTCGGC GCCTTCTTGG GAGGTGTCTG	660
	CCAGGGCTTC TCGTCCTCGT GGTGGAAGGA CAAGCACAAAC ACTCACCACG CCGCCCCCAA	720
35	CGTCCACGGC GAGGATCCCG ACATTGACAC CCACCCTCTG TTGACCTGGA GTGAGCATGC	780
	GTTGGAGATG TTCTCGGATG TCCCAGATGA GGAGCTGACC CGCATGTGGT CGCGTTTCAT	840
40	GGTCTGAAC CAGACCTGGT TTTACTTCCC CATTCTCTCG TTTGCCCCGC TCTCCTGGTG	900
	CCTCCAGTCC ATTCTCTTTG TGCTGCCTAA CGGTCAGGCC CACAAGCCCT CGGGCGCGCG	960
45	TGTGCCCATC TCGTTGGTCG AGCAGCTGTC GCTTGCGATG CACTGGACCT GGTACCTCGC	1020
	CACCATGTTC CTGTTTCATCA AGGATCCCGT CAACATGCTG GTGTACTTTT TGGTGTGCGA	1080
	GGCGGTGTGC GGAAACTTGT TGGCGATCGT GTTCTCGCTC AACCACAACG GTATGCCTGT	1140
50	GATCTCGAAG GAGGAGGCGG TCGATATGGA TTTCTTCACG AAGCAGATCA TCACGGGTCTG	1200
	TGATGTCCAC CCGGGTCTAT TTGCCAACTG GTTCACGGGT GGATTGAACT ATCAGATCGA	1260
	GCACCACTTG TTCCCTTCGA TGCCTCGCCA CAACTTTTCA AAGATCCAGC CTGCTGTCTG	1320
55	GACCTGTGTC AAAAAGTACA ATGTCCGATA CCACACCACC GGTATGATCG AGGGAAGTGC	1380
	AGAGGTCTTT AGCCGTCTGA ACGAGGTCTC CAAGGCTGCC TCCAAGATGG GTAAGGCGCA	1440
60	GTAAAAAAA AAACAAGGAC GTTTTTTTTC GCCAGTGCCT GTGCCTGTGC CTGCTTCCCT	1500
	TGTCAAGTCG AGCGTTTCTG GAAAGGATCG TTCAGTGCAG TATCATCATT CTCCTTTTAC	1560

CCCCGCTCA TATCTCATTC ATTCTCTTA TTAACAACCT TGTTCCTCCC TTCACCG 1617

5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

10

- (A) LENGTH: 457 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: peptide

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

25

Met Ala Ala Ala Pro Ser Val Arg Thr Phe Thr Arg Ala Glu Val Leu
1 5 10 15

Asn Ala Glu Ala Leu Asn Glu Gly Lys Lys Asp Ala Glu Ala Pro Phe
20 25 30

Leu Met Ile Ile Asp Asn Lys Val Tyr Asp Val Arg Glu Phe Val Pro
35 40 45

30

Asp His Pro Gly Gly Ser Val Ile Leu Thr His Val Gly Lys Asp Gly
50 55 60

35

Thr Asp Val Phe Asp Thr Phe His Pro Glu Ala Ala Trp Glu Thr Leu
65 70 75 80

Ala Asn Phe Tyr Val Gly Asp Ile Asp Glu Ser Asp Arg Asp Ile Lys
85 90 95

40

Asn Asp Asp Phe Ala Ala Glu Val Arg Lys Leu Arg Thr Leu Phe Gln
100 105 110

Ser Leu Gly Tyr Tyr Asp Ser Ser Lys Ala Tyr Tyr Ala Phe Lys Val
115 120 125

45

Ser Phe Asn Leu Cys Ile Trp Gly Leu Ser Thr Val Ile Val Ala Lys
130 135 140

50

Trp Gly Gln Thr Ser Thr Leu Ala Asn Val Leu Ser Ala Ala Leu Leu
145 150 155 160

Gly Leu Phe Trp Gln Gln Cys Gly Trp Leu Ala His Asp Phe Leu His
165 170 175

55

His Gln Val Phe Gln Asp Arg Phe Trp Gly Asp Leu Phe Gly Ala Phe
180 185 190

Leu Gly Gly Val Cys Gln Gly Phe Ser Ser Ser Trp Trp Lys Asp Lys
195 200 205

60

His Asn Thr His His Ala Ala Pro Asn Val His Gly Glu Asp Pro Asp
210 215 220

Ile Asp Thr His Pro Leu Leu Thr Trp Ser Glu His Ala Leu Glu Met
 225 230 235 240
 5 Phe Ser Asp Val Pro Asp Glu Glu Leu Thr Arg Met Trp Ser Arg Phe
 245 250 255
 Met Val Leu Asn Gln Thr Trp Phe Tyr Phe Pro Ile Leu Ser Phe Ala
 260 265 270
 10 Arg Leu Ser Trp Cys Leu Gln Ser Ile Leu Phe Val Leu Pro Asn Gly
 275 280 285
 Gln Ala His Lys Pro Ser Gly Ala Arg Val Pro Ile Ser Leu Val Glu
 290 295 300
 15 Gln Leu Ser Leu Ala Met His Trp Thr Trp Tyr Leu Ala Thr Met Phe
 305 310 315 320
 20 Leu Phe Ile Lys Asp Pro Val Asn Met Leu Val Tyr Phe Leu Val Ser
 325 330 335
 Gln Ala Val Cys Gly Asn Leu Leu Ala Ile Val Phe Ser Leu Asn His
 340 345 350
 25 Asn Gly Met Pro Val Ile Ser Lys Glu Glu Ala Val Asp Met Asp Phe
 355 360 365
 Phe Thr Lys Gln Ile Ile Thr Gly Arg Asp Val His Pro Gly Leu Phe
 370 375 380
 30 Ala Asn Trp Phe Thr Gly Gly Leu Asn Tyr Gln Ile Glu His His Leu
 385 390 395 400
 35 Phe Pro Ser Met Pro Arg His Asn Phe Ser Lys Ile Gln Pro Ala Val
 405 410 415
 Glu Thr Leu Cys Lys Lys Tyr Asn Val Arg Tyr His Thr Thr Gly Met
 420 425 430
 40 Ile Glu Gly Thr Ala Glu Val Phe Ser Arg Leu Asn Glu Val Ser Lys
 435 440 445
 Ala Ala Ser Lys Met Gly Lys Ala Gln
 450 455

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- 50 (A) LENGTH: 1488 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

60 GTCCCTGTC GCTGTCGGCA CACCCCATCC TCCCTCGCTC CCTCTGCGTT TGTCCTTGGC

60

CCACCGTCTC TCCTCCACCC TCCGAGACGA CTGCAACTGT AATCAGGAAC CGACAAATAC 120
 ACGATTTCTT TTTACTCAGC ACCAACTCAA AATCCTCAAC CGCAACCCTT TTTCAGGATG 180
 5 GCACCTCCCA AACTATATCGA TGCCGGTTTG ACCCAGCGTC ATATCAGCAC CTCGGCCCCA 240
 AACTCGGCCA AGCCTGCCTT CGAGCGCAAC TACCAGCTCC CCGAGTTCAC CATCAAGGAG 300
 10 ATCCGAGAGT GCATCCCTGC CCACTGCTTT GAGCGCTCCG GTCTCCGTGG TCTCTGCCAC 360
 GTTGCCATCG ATCTGACTTG GGCCTCGCTC TTGTTCTTGG CTGCGACCCA GATCGACAAG 420
 TTTGAGAATC CCTTGATCCG CTATTTGGCC TGGCCTGTTT ACTGGATCAT GCAGGGTATT 480
 15 GTCTGCACCG GTGTCTGGGT GCTGGCTCAC GAGTGTGGTC ATCAGTCCTT CTCGACCTCC 540
 AAGACCCTCA ACAACACAGT TGGTTGGATC TTGCACTCGA TGCTCTTGGT CCCCTACCAC 600
 TCCTGGAGAA TCTCGCACTC GAAGCACCAC AAGGCCACTG GCCATATGAC CAAGGACCAG 660
 20 GTCTTTGTGC CCAAGACCCG CTCCCAGGTT GGCTTGCCCTC CCAAGGAGAA CGCTGCTGCT 720
 GCCGTTGAGG AGGAGGACAT GTCCGTGCAC CTGGATGAGG AGGCTCCCAT TGTGACTTTG 780
 25 TTCTGGATGG TGATCCAGTT CTTGTTCCGA TGGCCCGCGT ACCTGATTAT GAACGCCTCT 840
 GGCCAAGACT ACGGCCGCTG GACCTCGCAC TTCCACACGT ACTCGCCCAT CTTTGAGCCC 900
 CGCAACTTTT TCGACATTAT TATCTCGGAC CTCGGTGTGT TGGCTGCCCT CGGTGCCCTG 960
 30 ATCTATGCCT CCATGCAGTT GTCGCTCTTG ACCGTACCA AGTACTATAT TGTCCTTAC 1020
 CTCTTTGTCA ACTTTTGGTT GGTCTGATC ACCTTCTTGC AGCACACCGA TCCCAAGCTG 1080
 35 CCCCATTACC GCGAGGGTGC CTGGAATTC CAGCGTGGAG CTCTTTGCAC CGTTGACCGC 1140
 TCGTTTGGCA AGTTCTTGGA CCATATGTTT CACGGCATTG TCCACACCCA TGTGGCCCAT 1200
 CACTTGTTCT CGCAAATGCC GTTCTACCAT GCTGAGGAAG CTACCTATCA TCTCAAGAAA 1260
 40 CTGCTGGGAG AGTACTATGT GTACGACCCA TCCCCGATCG TCGTTGCGGT CTGGAGGTCG 1320
 TTCCGTGAGT GCCGATTCGT GGAGGATCAG GGAGACGTGG TCTTTTTCAA GAAGTAAAAA 1380
 45 AAAAGACAAT GGACCACACA CAACCTTGTC TCTACAGACC TACGTATCAT GTAGCCATAC 1440
 CACTTCATAA AAGAACATGA GCTCTAGAGG CGTGTCATTC GCGCCTCC 1488

(2) INFORMATION FOR SEQ ID NO:4:

50

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 399 amino acids

(B) TYPE: amino acid

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(C) STRANDEDNESS: not relevant

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

	Met	Ala	Pro	Pro	Asn	Thr	Ile	Asp	Ala	Gly	Leu	Thr	Gln	Arg	His	Ile	
	1				5					10					15		
5	Ser	Thr	Ser	Ala	Pro	Asn	Ser	Ala	Lys	Pro	Ala	Phe	Glu	Arg	Asn	Tyr	
				20					25					30			
	Gln	Leu	Pro	Glu	Phe	Thr	Ile	Lys	Glu	Ile	Arg	Glu	Cys	Ile	Pro	Ala	
10			35					40					45				
	His	Cys	Phe	Glu	Arg	Ser	Gly	Leu	Arg	Gly	Leu	Cys	His	Val	Ala	Ile	
		50					55					60					
15	Asp	Leu	Thr	Trp	Ala	Ser	Leu	Leu	Phe	Leu	Ala	Ala	Thr	Gln	Ile	Asp	
	65					70					75					80	
	Lys	Phe	Glu	Asn	Pro	Leu	Ile	Arg	Tyr	Leu	Ala	Trp	Pro	Val	Tyr	Trp	
				85						90					95		
20	Ile	Met	Gln	Gly	Ile	Val	Cys	Thr	Gly	Val	Trp	Val	Leu	Ala	His	Glu	
				100					105					110			
	Cys	Gly	His	Gln	Ser	Phe	Ser	Thr	Ser	Lys	Thr	Leu	Asn	Asn	Thr	Val	
25			115					120					125				
	Gly	Trp	Ile	Leu	His	Ser	Met	Leu	Leu	Val	Pro	Tyr	His	Ser	Trp	Arg	
		130					135					140					
30	Ile	Ser	His	Ser	Lys	His	His	Lys	Ala	Thr	Gly	His	Met	Thr	Lys	Asp	
	145					150					155					160	
	Gln	Val	Phe	Val	Pro	Lys	Thr	Arg	Ser	Gln	Val	Gly	Leu	Pro	Pro	Lys	
					165					170					175		
35	Glu	Asn	Ala	Ala	Ala	Ala	Val	Gln	Glu	Glu	Asp	Met	Ser	Val	His	Leu	
				180					185					190			
	Asp	Glu	Glu	Ala	Pro	Ile	Val	Thr	Leu	Phe	Trp	Met	Val	Ile	Gln	Phe	
40			195					200					205				
	Leu	Phe	Gly	Trp	Pro	Ala	Tyr	Leu	Ile	Met	Asn	Ala	Ser	Gly	Gln	Asp	
		210					215					220					
45	Tyr	Gly	Arg	Trp	Thr	Ser	His	Phe	His	Thr	Tyr	Ser	Pro	Ile	Phe	Glu	
	225					230					235					240	
	Pro	Arg	Asn	Phe	Phe	Asp	Ile	Ile	Ile	Ser	Asp	Leu	Gly	Val	Leu	Ala	
				245						250					255		
50	Ala	Leu	Gly	Ala	Leu	Ile	Tyr	Ala	Ser	Met	Gln	Leu	Ser	Leu	Leu	Thr	
				260					265					270			
	Val	Thr	Lys	Tyr	Tyr	Ile	Val	Pro	Tyr	Leu	Phe	Val	Asn	Phe	Trp	Leu	
55			275					280					285				
	Val	Leu	Ile	Thr	Phe	Leu	Gln	His	Thr	Asp	Pro	Lys	Leu	Pro	His	Tyr	
		290					295					300					
60	Arg	Glu	Gly	Ala	Trp	Asn	Phe	Gln	Arg	Gly	Ala	Leu	Cys	Thr	Val	Asp	
	305					310					315					320	

Arg Ser Phe Gly Lys Phe Leu Asp His Met Phe His Gly Ile Val His
 325 330 335
 5 Thr His Val Ala His His Leu Phe Ser Gln Met Pro Phe Tyr His Ala
 340 345 350
 Glu Glu Ala Thr Tyr His Leu Lys Lys Leu Leu Gly Glu Tyr Tyr Val
 355 360 365
 10 Tyr Asp Pro Ser Pro Ile Val Val Ala Val Trp Arg Ser Phe Arg Glu
 370 375 380
 Cys Arg Phe Val Glu Asp Gln Gly Asp Val Val Phe Phe Lys Lys
 385 390 395

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1483 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

30 GCTTCCTCCA GTTCATCCTC CATTTGCGCA CCTGCATTCT TTACGACCGT TAAGCAAGAT 60
 GGGAACGGAC CAAGGAAAAA CCTTCACCTG GGAAGAGCTG GCGGCCCATTA ACACCAAGGA 120
 35 CGACCTACTC TTGGCCATCC GCGGCAGGGT GTACGATGTC ACAAAGTTCT TGAGCCGCCA 180
 TCCTGGTGGA GTGGACACTC TCCTGCTCGG AGCTGGCCGA GATGTTACTC CGGTCTTTGA 240
 40 GATGTATCAC GCGTTTGGGG CTGCAGATGC CATTATGAAG AAGTACTATG TCGGTACACT 300
 GGTCTCGAAT GAGCTGCCCA TCTTCCCGGA GCCAACGGTG TTCCACAAAA CCATCAAGAC 360
 GAGAGTCGAG GGCTACTTTA CGGATCGGAA CATTGATCCC AAGAATAGAC CAGAGATCTG 420
 45 GGGACGATAC GCTCTTATCT TTGGATCCTT GATCGCTTCC TACTACGCGC AGCTCTTTGT 480
 GCCTTTCGTT GTCGAACGCA CATGGCTTCA GGTGGTGTTT GCAATCATCA TGGGATTTGC 540
 50 GTGCGCACAA GTCGGACTCA ACCCTCTTCA TGATGCGTCT CACTTTTCAG TGACCCACAA 600
 CCCCCTGTC TGGAAGATTG TGGGAGCCAC GCACGACTTT TTCAACGGAG CATCGTACCT 660
 GGTGTGGATG TACCAACATA TGCTCGGCCA TCACCCCTAC ACCAACATTG CTGGAGCAGA 720
 55 TCCCGACGTG TCGACGTCTG AGCCCGATGT TCGTCGTATC AAGCCCAACC AAAAGTGGTT 780
 TGTCACCAC ATCAACCAGC ACATGTTTGT TCCTTTCCTG TACGGACTGC TGGCGTTCAA 840
 GGTGCGCATT CAGGACATCA ACATTTTGTA CTTTGTCAAG ACCAATGACG CTATTCGTGT 900
 60 CAATCCCATC TCGACATGGC AACTGTGAT GTTCTGGGGC GGCAAGGCTT TCTTTGTCTG 960

GTATCGCCTG ATTGTTCCCC TGCAGTATCT GCCCCTGGGC AAGGTGCTGC TCTTGTTTAC 1020
 GGTCGCGGAC ATGGTGTCGT CTTACTGGCT GGCGCTGACC TTCCAGGCGA ACCACGTTGT 1080
 5 TGAGGAAGTT CAGTGGCCGT TGCCTGACGA GAACGGGATC ATCCAAAAGG ACTGGGCAGC 1140
 TATGCAGGTC GAGACTACGC AGGATTACGC ACACGATTCT CACCTCTGGA CCAGCATCAC 1200
 10 TGGCAGCTTG AACTACCAGG CTGTGCACCA TCTGTTCCCC AACGTGTCGC AGCACCATTA 1260
 TCCCGATATT CTGGCCATCA TCAAGAACAC CTGCAGCGAG TACAAGGTTT CATACTTGT 1320
 CAAGGATACG TTTTGGCAAG CATTGCTTC ACATTGGAG CACTTGCGTG TTCTTGGACT 1380
 15 CCGTCCCAAG GAAGAGTAGA AGAAAAAAG CGCCGAATGA AGTATTGCCC CCTTTTCTC 1440
 CAAGAATGGC AAAAGGAGAT CAAGTGGACA TTCTCTATGA AGA 1483

20 (2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 446 amino acids
 (B) TYPE: amino acid
 25 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

35 Met Gly Thr Asp Gln Gly Lys Thr Phe Thr Trp Glu Glu Leu Ala Ala
 1 5 10 15
 His Asn Thr Lys Asp Asp Leu Leu Leu Ala Ile Arg Gly Arg Val Tyr
 20 25 30
 40 Asp Val Thr Lys Phe Leu Ser Arg His Pro Gly Gly Val Asp Thr Leu
 35 40 45
 Leu Leu Gly Ala Gly Arg Asp Val Thr Pro Val Phe Glu Met Tyr His
 50 55 60
 45 Ala Phe Gly Ala Ala Asp Ala Ile Met Lys Lys Tyr Tyr Val Gly Thr
 65 70 75 80
 50 Leu Val Ser Asn Glu Leu Pro Ile Phe Pro Glu Pro Thr Val Phe His
 85 90 95
 Lys Thr Ile Lys Thr Arg Val Glu Gly Tyr Phe Thr Asp Arg Asn Ile
 100 105 110
 55 Asp Pro Lys Asn Arg Pro Glu Ile Trp Gly Arg Tyr Ala Leu Ile Phe
 115 120 125
 Gly Ser Leu Ile Ala Ser Tyr Tyr Ala Gln Leu Phe Val Pro Phe Val
 130 135 140
 60 Val Glu Arg Thr Trp Leu Gln Val Val Phe Ala Ile Ile Met Gly Phe
 145 150 155 160

	Ala Cys Ala Gln Val Gly Leu Asn Pro Leu His Asp Ala Ser His Phe	
	165	170 175
5	Ser Val Thr His Asn Pro Thr Val Trp Lys Ile Leu Gly Ala Thr His	
	180	185 190
	Asp Phe Phe Asn Gly Ala Ser Tyr Leu Val Trp Met Tyr Gln His Met	
	195	200 205
10	Leu Gly His His Pro Tyr Thr Asn Ile Ala Gly Ala Asp Pro Asp Val	
	210	215 220
	Ser Thr Ser Glu Pro Asp Val Arg Arg Ile Lys Pro Asn Gln Lys Trp	
	225	230 235 240
	Phe Val Asn His Ile Asn Gln His Met Phe Val Pro Phe Leu Tyr Gly	
	245	250 255
20	Leu Leu Ala Phe Lys Val Arg Ile Gln Asp Ile Asn Ile Leu Tyr Phe	
	260	265 270
	Val Lys Thr Asn Asp Ala Ile Arg Val Asn Pro Ile Ser Thr Trp His	
	275	280 285
25	Thr Val Met Phe Trp Gly Gly Lys Ala Phe Phe Val Trp Tyr Arg Leu	
	290	295 300
	Ile Val Pro Leu Gln Tyr Leu Pro Leu Gly Lys Val Leu Leu Leu Phe	
	305	310 315 320
	Thr Val Ala Asp Met Val Ser Ser Tyr Trp Leu Ala Leu Thr Phe Gln	
	325	330 335
35	Ala Asn His Val Val Glu Glu Val Gln Trp Pro Leu Pro Asp Glu Asn	
	340	345 350
	Gly Ile Ile Gln Lys Asp Trp Ala Ala Met Gln Val Glu Thr Thr Gln	
	355	360 365
40	Asp Tyr Ala His Asp Ser His Leu Trp Thr Ser Ile Thr Gly Ser Leu	
	370	375 380
	Asn Tyr Gln Ala Val His His Leu Phe Pro Asn Val Ser Gln His His	
	385	390 395 400
	Tyr Pro Asp Ile Leu Ala Ile Ile Lys Asn Thr Cys Ser Glu Tyr Lys	
	405	410 415
50	Val Pro Tyr Leu Val Lys Asp Thr Phe Trp Gln Ala Phe Ala Ser His	
	420	425 430 435
	Leu Glu His Leu Arg Val Leu Gly Leu Arg Pro Lys Glu Glu	
	435	440 445

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- 60
- (A) LENGTH: 355 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: not relevant
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

10	Glu Val Arg Lys Leu Arg Thr Leu Phe Gln Ser Leu Gly Tyr Tyr Asp	1	5	10	15
	Ser Ser Lys Ala Tyr Tyr Ala Phe Lys Val Ser Phe Asn Leu Cys Ile	20	25	30	
15	Trp Gly Leu Ser Thr Val Ile Val Ala Lys Trp Gly Gln Thr Ser Thr	35	40	45	
	Leu Ala Asn Val Leu Ser Ala Ala Leu Leu Gly Leu Phe Trp Gln Gln	50	55	60	
20	Cys Gly Trp Leu Ala His Asp Phe Leu His His Gln Val Phe Gln Asp	65	70	75	80
	Arg Phe Trp Gly Asp Leu Phe Gly Ala Phe Leu Gly Gly Val Cys Gln	85	90	95	
25	Gly Phe Ser Ser Ser Trp Trp Lys Asp Lys His Asn Thr His His Ala	100	105	110	
30	Ala Pro Asn Val His Gly Glu Asp Pro Asp Ile Asp Thr His Pro Leu	115	120	125	
	Leu Thr Trp Ser Glu His Ala Leu Glu Met Phe Ser Asp Val Pro Asp	130	135	140	
35	Glu Glu Leu Thr Arg Met Trp Ser Arg Phe Met Val Leu Asn Gln Thr	145	150	155	160
40	Trp Phe Tyr Phe Pro Ile Leu Ser Phe Ala Arg Leu Ser Trp Cys Leu	165	170	175	
	Gln Ser Ile Leu Phe Val Leu Pro Asn Gly Gln Ala His Lys Pro Ser	180	185	190	
45	Gly Ala Arg Val Pro Ile Ser Leu Val Glu Gln Leu Ser Leu Ala Met	195	200	205	
	His Trp Thr Trp Tyr Leu Ala Thr Met Phe Leu Phe Ile Lys Asp Pro	210	215	220	
50	Val Asn Met Leu Val Tyr Phe Leu Val Ser Gln Ala Val Cys Gly Asn	225	230	235	240
55	Leu Leu Ala Ile Val Phe Ser Leu Asn His Asn Gly Met Pro Val Ile	245	250	255	
	Ser Lys Glu Glu Ala Val Asp Met Asp Phe Phe Thr Lys Gln Ile Ile	260	265	270	
60	Thr Gly Arg Asp Val His Pro Gly Leu Phe Ala Asn Trp Phe Thr Gly	275	280	285	

Gly Leu Asn Tyr Gln Ile Glu His His Leu Phe Pro Ser Met Pro Arg
 290 295 300
 5 His Asn Phe Ser Lys Ile Gln Pro Ala Val Glu Thr Leu Cys Lys Lys
 305 310 315 320
 Tyr Asn Val Arg Tyr His Thr Thr Gly Met Ile Glu Gly Thr Ala Glu
 325 330 335
 10 Val Phe Ser Arg Leu Asn Glu Val Ser Lys Ala Ala Ser Lys Met Gly
 340 345 350
 Lys Ala Gln
 15 355

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 104 amino acids
 20 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: peptide

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Val Thr Leu Tyr Thr Leu Ala Phe Val Ala Ala Asn Ser Leu Gly Val
 1 5 10 15
 35 Leu Tyr Gly Val Leu Ala Cys Pro Ser Val Xaa Pro His Gln Ile Ala
 20 25 30
 Ala Gly Leu Leu Gly Leu Leu Trp Ile Gln Ser Ala Tyr Ile Gly Xaa
 35 40 45
 40 Asp Ser Gly His Tyr Val Ile Met Ser Asn Lys Ser Asn Asn Xaa Phe
 50 55 60
 45 Ala Gln Leu Leu Ser Gly Asn Cys Leu Thr Gly Ile Ile Ala Trp Trp
 65 70 75 80
 Lys Trp Thr His Asn Ala His His Leu Ala Cys Asn Ser Leu Asp Tyr
 85 90 95
 50 Gly Pro Asn Leu Gln His Ile Pro
 100

(2) INFORMATION FOR SEQ ID NO:9:

55 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 252 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

60 (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

5 Gly Val Leu Tyr Gly Val Leu Ala Cys Thr Ser Val Phe Ala His Gln
 1 5 10 15
 Ile Ala Ala Ala Leu Leu Gly Leu Leu Trp Ile Gln Ser Ala Tyr Ile
 20 25 30
 10 Gly His Asp Ser Gly His Tyr Val Ile Met Ser Asn Lys Ser Tyr Asn
 35 40 45
 15 Arg Phe Ala Gln Leu Leu Ser Gly Asn Cys Leu Thr Gly Ile Ser Ile
 50 55 60
 Ala Trp Trp Lys Trp Thr His Asn Ala His His Leu Ala Cys Asn Ser
 65 70 75 80
 20 Leu Asp Tyr Asp Pro Asp Leu Gln His Ile Pro Val Phe Ala Val Ser
 85 90 95
 Thr Lys Phe Phe Ser Ser Leu Thr Ser Arg Phe Tyr Asp Arg Lys Leu
 100 105 110
 25 Thr Phe Gly Pro Val Ala Arg Phe Leu Val Ser Tyr Gln His Phe Thr
 115 120 125
 30 Tyr Tyr Pro Val Asn Cys Phe Gly Arg Ile Asn Leu Phe Ile Gln Thr
 130 135 140
 Phe Leu Leu Leu Phe Ser Lys Arg Glu Val Pro Asp Arg Ala Leu Asn
 145 150 155 160
 35 Phe Ala Gly Ile Leu Val Phe Trp Thr Trp Phe Pro Leu Leu Val Ser
 165 170 175
 Cys Leu Pro Asn Trp Pro Glu Arg Phe Phe Phe Val Phe Thr Ser Phe
 180 185 190
 40 Thr Val Thr Ala Leu Gln His Ile Gln Phe Thr Leu Asn His Phe Ala
 195 200 205
 45 Ala Asp Val Tyr Val Gly Pro Pro Thr Gly Ser Asp Trp Phe Glu Lys
 210 215 220
 Gln Ala Ala Gly Thr Ile Asp Ile Ser Cys Arg Ser Tyr Met Asp Trp
 225 230 235 240
 50 Phe Phe Gly Gly Leu Gln Phe Gln Leu Glu His His
 245 250

(2) INFORMATION FOR SEQ ID NO:10:

- 55 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 125 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear
 60 (ii) MOLECULE TYPE: peptide

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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Gly Xaa Xaa Asn Phe Ala Gly Ile Leu Val Phe Trp Thr Trp Phe Pro
 1 5 10 15
 Leu Leu Val Ser Cys Leu Pro Asn Trp Pro Glu Arg Phe Xaa Phe Val
 20 25 30
 Phe Thr Gly Phe Thr Val Thr Ala Leu Gln His Ile Gln Phe Thr Leu
 35 40 45
 Asn His Phe Ala Ala Asp Val Tyr Val Gly Pro Pro Thr Gly Ser Asp
 50 55 60
 Trp Phe Glu Lys Gln Ala Ala Gly Thr Ile Asp Ile Ser Cys Arg Ser
 65 70 75 80
 Tyr Met Asp Trp Phe Phe Cys Gly Leu Gln Phe Gln Leu Glu His His
 85 90 95
 Leu Phe Pro Arg Leu Pro Arg Cys His Leu Arg Lys Val Ser Pro Val
 100 105 110
 Gly Gln Arg Gly Phe Gln Arg Lys Xaa Asn Leu Ser Xaa
 115 120 125

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 131 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Pro Ala Thr Glu Val Gly Gly Leu Ala Trp Met Ile Thr Phe Tyr Val
 1 5 10 15
 Arg Phe Phe Leu Thr Tyr Val Pro Leu Leu Gly Leu Lys Ala Phe Leu
 20 25 30
 Gly Leu Phe Phe Ile Val Arg Phe Leu Glu Ser Asn Trp Phe Val Trp
 35 40 45
 Val Thr Gln Met Asn His Ile Pro Met His Ile Asp His Asp Arg Asn
 50 55 60
 Met Asp Trp Val Ser Thr Gln Leu Gln Ala Thr Cys Asn Val His Lys
 65 70 75 80
 Ser Ala Phe Asn Asp Trp Phe Ser Gly His Leu Asn Phe Gln Ile Glu
 85 90 95

His His Leu Phe Pro Thr Met Pro Arg His Asn Tyr His Xaa Val Ala
100 105 110

5 Pro Leu Val Gln Ser Leu Cys Ala Lys His Gly Ile Glu Tyr Gln Ser
115 120 125

Lys Pro Leu
130

10 (2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

15 (A) LENGTH: 87 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

25 Cys Ser Pro Lys Ser Ser Pro Thr Arg Asn Met Thr Pro Ser Pro Phe
1 5 10 15

Ile Asp Trp Leu Trp Gly Gly Leu Asn Tyr Gln Ile Glu His His Leu
20 25 30

30 Phe Pro Thr Met Pro Arg Cys Asn Leu Asn Arg Cys Met Lys Tyr Val
35 40 45

35 Lys Glu Trp Cys Ala Glu Asn Asn Leu Pro Tyr Leu Val Asp Asp Tyr
50 55 60

Phe Val Gly Tyr Asn Leu Asn Leu Gln Gln Leu Lys Asn Met Ala Glu
65 70 75 80

40 Leu Val Gln Ala Lys Ala Ala
85

(2) INFORMATION FOR SEQ ID NO:13:

45 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 143 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
50 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

60 Arg His Glu Ala Ala Arg Gly Gly Thr Arg Leu Ala Tyr Met Leu Val
1 5 10 15

Cys Met Gln Trp Thr Asp Leu Leu Trp Ala Ala Ser Phe Tyr Ser Arg
20 25 30

5 Phe Phe Leu Ser Tyr Ser Pro Phe Tyr Gly Ala Thr Gly Thr Leu Leu
 35 40 45
 Leu Phe Val Ala Val Arg Val Leu Glu Ser His Trp Phe Val Trp Ile
 50 55 60
 10 Thr Gln Met Asn His Ile Pro Lys Glu Ile Gly His Glu Lys His Arg
 65 70 75 80
 Asp Trp Ala Ser Ser Gln Leu Ala Ala Thr Cys Asn Val Glu Pro Ser
 85 90 95
 15 Leu Phe Ile Asp Trp Phe Ser Gly His Leu Asn Phe Gln Ile Glu His
 100 105 110
 His Leu Phe Pro Thr Met Thr Arg His Asn Tyr Arg Xaa Val Ala Pro
 115 120 125
 20 Leu Val Lys Ala Phe Cys Ala Lys His Gly Leu His Tyr Glu Val
 130 135 140

(2) INFORMATION FOR SEQ ID NO:14:

25 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 186 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 30 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

40 Leu His His Thr Tyr Thr Asn Ile Ala Gly Ala Asp Pro Asp Val Ser
 1 5 10 15
 Thr Ser Glu Pro Asp Val Arg Arg Ile Lys Pro Asn Gln Lys Trp Phe
 20 25 30
 45 Val Asn His Ile Asn Gln His Met Phe Val Pro Phe Leu Tyr Gly Leu
 35 40 45
 Leu Ala-Phe Lys Val Arg Ile Gln Asp Ile Asn Ile Leu Tyr Phe Val
 50 55 60
 50 Lys Thr Asn Asp Ala Ile Arg Val Asn Pro Ile Ser Thr Trp His Thr
 65 70 75 80
 Val Met Phe Trp Gly Gly Lys Ala Phe Phe Val Trp Tyr Arg Leu Ile
 85 90 95
 55 Val Pro Leu Gln Tyr Leu Pro Leu Gly Lys Val Leu Leu Leu Phe Thr
 100 105 110
 60 Val Ala Asp Met Val Ser Ser Tyr Trp Leu Ala Leu Thr Phe Gln Ala
 115 120 125

Asn Tyr Val Val Glu Glu Val Gln Trp Pro Leu Pro Asp Glu Asn Gly
 130 135 140

5 Ile Ile Gln Lys Asp Trp Ala Ala Met Gln Val Glu Thr Thr Gln Asp
 145 150 155 160

Tyr Ala His Asp Ser His Leu Trp Thr Ser Ile Thr Gly Ser Leu Asn
 165 170 175

10 Tyr Gln Xaa Val His His Leu Phe Pro His
 180 185

(2) INFORMATION FOR SEQ ID NO:15:

15 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 5 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: peptide

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

His Xaa Xaa His His
 1 5

30 (2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 446 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: peptide

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

45 Met Ala Ala Gln Ile Lys Lys Tyr Ile Thr Ser Asp Glu Leu Lys Asn
 1 5 10 15

50 His Asp Lys Pro Gly Asp Leu Trp Ile Ser Ile Gln Gly Lys Ala Tyr
 20 25 30

Asp Val Ser Asp Trp Val Lys Asp His Pro Gly Gly Ser Phe Pro Leu
 35 40 45

55 Lys Ser Leu Ala Gly Gln Glu Val Thr Asp Ala Phe Val Ala Phe His
 50 55 60

60 Pro Ala Ser Thr Trp Lys Asn Leu Asp Lys Phe Phe Thr Gly Tyr Tyr
 65 70 75 80

Leu Lys Asp Tyr Ser Val Ser Glu Val Ser Lys Val Tyr Arg Lys Leu
 85 90 95

	Val	Phe	Glu	Phe	Ser	Lys	Met	Gly	Leu	Tyr	Asp	Lys	Lys	Gly	His	Ile	
				100					105					110			
5	Met	Phe	Ala	Thr	Leu	Cys	Phe	Ile	Ala	Met	Leu	Phe	Ala	Met	Ser	Val	
			115					120					125				
	Tyr	Gly	Val	Leu	Phe	Cys	Glu	Gly	Val	Leu	Val	His	Leu	Phe	Ser	Gly	
10		130					135					140					
	Cys	Leu	Met	Gly	Phe	Leu	Trp	Ile	Gln	Ser	Gly	Trp	Ile	Gly	His	Asp	
	145					150					155					160	
	Ala	Gly	His	Tyr	Met	Val	Val	Ser	Asp	Ser	Arg	Leu	Asn	Lys	Phe	Met	
15					165					170					175		
	Gly	Ile	Phe	Ala	Ala	Asn	Cys	Leu	Ser	Gly	Ile	Ser	Ile	Gly	Trp	Trp	
				180					185					190			
20	Lys	Trp	Asn	His	Asn	Ala	His	His	Ile	Ala	Cys	Asn	Ser	Leu	Glu	Tyr	
			195					200					205				
	Asp	Pro	Asp	Leu	Gln	Tyr	Ile	Pro	Phe	Leu	Val	Val	Ser	Ser	Lys	Phe	
25		210					215					220					
	Phe	Gly	Ser	Leu	Thr	Ser	His	Phe	Tyr	Glu	Lys	Arg	Leu	Thr	Phe	Asp	
	225					230					235					240	
	Ser	Leu	Ser	Arg	Phe	Phe	Val	Ser	Tyr	Gln	His	Trp	Thr	Phe	Tyr	Pro	
30					245					250					255		
	Ile	Met	Cys	Ala	Ala	Arg	Leu	Asn	Met	Tyr	Val	Gln	Ser	Leu	Ile	Met	
				260					265					270			
35	Leu	Leu	Thr	Lys	Arg	Asn	Val	Ser	Tyr	Arg	Ala	Gln	Glu	Leu	Leu	Gly	
			275					280					285				
	Cys	Leu	Val	Phe	Ser	Ile	Trp	Tyr	Pro	Leu	Leu	Val	Ser	Cys	Leu	Pro	
40		290					295					300					
	Asn	Trp	Gly	Glu	Arg	Ile	Met	Phe	Val	Ile	Ala	Ser	Leu	Ser	Val	Thr	
	305					310					315					320	
	Gly	Met	Gln	Gln	Val	Gln	Phe	Ser	Leu	Asn	His	Phe	Ser	Ser	Ser	Val	
45					325					330					335		
	Tyr	Val	Gly	Lys	Pro	Lys	Gly	Asn	Asn	Trp	Phe	Glu	Lys	Gln	Thr	Asp	
				340					345					350			
50	Gly	Thr	Leu	Asp	Ile	Ser	Cys	Pro	Pro	Trp	Met	Asp	Trp	Phe	His	Gly	
			355					360					365				
	Gly	Leu	Gln	Phe	Gln	Ile	Glu	His	His	Leu	Phe	Pro	Lys	Met	Pro	Arg	
55		370					375					380					
	Cys	Asn	Leu	Arg	Lys	Ile	Ser	Pro	Tyr	Val	Ile	Glu	Leu	Cys	Lys	Lys	
	385					390					395					400	
60	His	Asn	Leu	Pro	Tyr	Asn	Tyr	Ala	Ser	Phe	Ser	Lys	Ala	Asn	Glu	Met	
					405					410					415		

Thr Leu Arg Thr Leu Arg Asn Thr Ala Leu Gln Ala Arg Asp Ile Thr
420 425 430

5 Lys Pro Leu Pro Lys Asn Leu Val Trp Glu Ala Leu His Thr
435 440 445

(2) INFORMATION FOR SEQ ID NO:17:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 359 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: peptide

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Met Leu Thr Ala Glu Arg Ile Lys Phe Thr Gln Lys Arg Gly Phe Arg
1 5 10 15

25 Arg Val Leu Asn Gln Arg Val Asp Ala Tyr Phe Ala Glu His Gly Leu
20 25 30

Thr Gln Arg Asp Asn Pro Ser Met Tyr Leu Lys Thr Leu Ile Ile Val
35 40 45

30 Leu Trp Leu Phe Ser Ala Trp Ala Phe Val Leu Phe Ala Pro Val Ile
50 55 60

35 Phe Pro Val Arg Leu Leu Gly Cys Met Val Leu Ala Ile Ala Leu Ala
65 70 75 80

Ala Phe Ser Phe Asn Val Gly His Asp Ala Asn His Asn Ala Tyr Ser
85 90 95

40 Ser Asn Pro His Ile Asn Arg Val Leu Gly Met Thr Tyr Asp Phe Val
100 105 110

45 Gly Leu Ser Ser Phe Leu Trp Arg Tyr Arg His Asn Tyr Leu His His
115 120 125

Thr Tyr Thr Asn Ile Leu Gly His Asp Val Glu Ile His Gly Asp Gly
130 135 140

50 Ala Val Arg Met Ser Pro Glu Gln Glu His Val Gly Ile Tyr Arg Phe
145 150 155 160

Gln Gln Phe Tyr Ile Trp Gly Leu Tyr Leu Phe Ile Pro Phe Tyr Trp
165 170 175

55 Phe Leu Tyr Asp Val Tyr Leu Val Leu Asn Lys Gly Lys Tyr His Asp
180 185 190

His Lys Ile Pro Pro Phe Gln Pro Leu Glu Leu Ala Ser Leu Leu Gly
195 200 205

60 Ile Lys Leu Leu Trp Leu Gly Tyr Val Phe Gly Leu Pro Leu Ala Leu
210 215 220

Gly Phe Ser Ile Pro Glu Val Leu Ile Gly Ala Ser Val Thr Tyr Met
 225 230 235 240
 5 Thr Tyr Gly Ile Val Val Cys Thr Ile Phe Met Leu Ala His Val Leu
 245 250 255
 Glu Ser Thr Glu Phe Leu Thr Pro Asp Gly Glu Ser Gly Ala Ile Asp
 260 265 270
 10 Asp Glu Trp Ala Ile Cys Gln Ile Arg Thr Thr Ala Asn Phe Ala Thr
 275 280 285
 Asn Asn Pro Phe Trp Asn Trp Phe Cys Gly Gly Leu Asn His Gln Val
 290 295 300
 15 Thr His His Leu Phe Pro Asn Ile Cys His Ile His Tyr Pro Gln Leu
 305 310 315 320
 20 Glu Asn Ile Ile Lys Asp Val Cys Gln Glu Phe Gly Val Glu Tyr Lys
 325 330 335
 Val Tyr Pro Thr Phe Lys Ala Ala Ile Ala Ser Asn Tyr Arg Trp Leu
 340 345 350
 25 Glu Ala Met Gly Lys Ala Ser
 355

(2) INFORMATION FOR SEQ ID NO:18:

30 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 365 amino acids
 (B) TYPE: amino acid
 35 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

45 Met Thr Ser Thr Thr Ser Lys Val Thr Phe Gly Lys Ser Ile Gly Phe
 1 5 10 15
 Arg Lys Glu Leu Asn Arg Arg Val Asn Ala Tyr Leu Glu Ala Glu Asn
 20 25 30
 50 Ile Ser Pro Arg Asp Asn Pro Pro Met Tyr Leu Lys Thr Ala Ile Ile
 35 40 45
 Leu Ala Trp Val Val Ser Ala Trp Thr Phe Val Val Phe Gly Pro Asp
 50 55 60
 55 Val Leu Trp Met Lys Leu Leu Gly Cys Ile Val Leu Gly Phe Gly Val
 65 70 75 80
 60 Ser Ala Val Gly Phe Asn Ile Ser His Asp Gly Asn His Gly Gly Tyr
 85 90 95

	Ser	Lys	Tyr	Gln	Trp	Val	Asn	Tyr	Leu	Ser	Gly	Leu	Thr	His	Asp	Ala	
				100					105						110		
5	Ile	Gly	Val	Ser	Ser	Tyr	Leu	Trp	Lys	Phe	Arg	His	Asn	Val	Leu	His	
			115					120					125				
	His	Thr	Tyr	Thr	Asn	Ile	Leu	Gly	His	Asp	Val	Glu	Ile	His	Gly	Asp	
		130					135					140					
10	Glu	Leu	Val	Arg	Met	Ser	Pro	Ser	Met	Glu	Tyr	Arg	Trp	Tyr	His	Arg	
	145					150					155					160	
	Tyr	Gln	His	Trp	Phe	Ile	Trp	Phe	Val	Tyr	Pro	Phe	Ile	Pro	Tyr	Tyr	
				165						170					175		
15	Trp	Ser	Ile	Ala	Asp	Val	Gln	Thr	Met	Leu	Phe	Lys	Arg	Gln	Tyr	His	
			180						185					190			
	Asp	His	Glu	Ile	Pro	Ser	Pro	Thr	Trp	Val	Asp	Ile	Ala	Thr	Leu	Leu	
20			195					200					205				
	Ala	Phe	Lys	Ala	Phe	Gly	Val	Ala	Val	Phe	Leu	Ile	Ile	Pro	Ile	Ala	
		210					215					220					
25	Val	Gly	Tyr	Ser	Pro	Leu	Glu	Ala	Val	Ile	Gly	Ala	Ser	Ile	Val	Tyr	
	225					230					235					240	
	Met	Thr	His	Gly	Leu	Val	Ala	Cys	Val	Val	Phe	Met	Leu	Ala	His	Val	
				245						250					255		
30	Ile	Glu	Pro	Ala	Glu	Phe	Leu	Asp	Pro	Asp	Asn	Leu	His	Ile	Asp	Asp	
			260						265					270			
	Glu	Trp	Ala	Ile	Ala	Gln	Val	Lys	Thr	Thr	Val	Asp	Phe	Ala	Pro	Asn	
35			275					280					285				
	Asn	Thr	Ile	Ile	Asn	Trp	Tyr	Val	Gly	Gly	Leu	Asn	Tyr	Gln	Thr	Val	
		290					295					300					
40	His	His	Leu	Phe	Pro	His	Ile	Cys	His	Ile	His	Tyr	Pro	Lys	Ile	Ala	
	305					310					315					320	
	Pro	Ile	Leu	Ala	Glu	Val	Cys	Glu	Glu	Phe	Gly	Val	Asn	Tyr	Ala	Val	
45				325						330					335		
	His	Gln	Thr	Phe	Phe	Gly	Ala	Leu	Ala	Ala	Asn	Tyr	Ser	Trp	Leu	Lys	
			340					345						350			
50	Lys	Met	Ser	Ile	Asn	Pro	Glu	Thr	Lys	Ala	Ile	Glu	Gln				
		355						360					365				

(2) INFORMATION FOR SEQ ID NO:19:

- | | |
|----|--|
| 55 | (i) SEQUENCE CHARACTERISTICS: |
| | (A) LENGTH: 35 base pairs |
| | (B) TYPE: nucleic acid |
| | (C) STRANDEDNESS: single |
| | (D) TOPOLOGY: linear |
| 60 | (ii) MOLECULE TYPE: other nucleic acid |

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

5 CCAAGCTTCT GCAGGAGCTC TTTTTTTTTT TTTT 35

(2) INFORMATION FOR SEQ ID NO:20:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic oligonucleotide"

20 (ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 21
(D) OTHER INFORMATION: /number= 1
/note= "N=Inosine or Cytosine"

25 (ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 27
(D) OTHER INFORMATION: /number= 2
/note= "N=Inosine or Cytosine"

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

35 CUACUACUAC UACAYCAYAC NTAYACNAAY AT 32

(2) INFORMATION FOR SEQ ID NO:21:

40 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic oligonucleotide"

50 (ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 13
(D) OTHER INFORMATION: /number= 1
/note= "N=Inosine or Cytosine"

55 (ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 19
(D) OTHER INFORMATION: /number= 2
/note= "N=Inosine or Cytosine"

60

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CAUCAUCAUC AUNGGRAANA RRTGRTG

27

(2) INFORMATION FOR SEQ ID NO:22:

- 5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
10 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CUACUACUAC UAGGAGTCCT CTACGGTGTT TTG

33

20

(2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
25 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

35 CAUCAUCAUC AUATGATGCT CAAGCTGAAA CTG

33

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
40 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide

45

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Gln Xaa Xaa His His
1 5

55

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
60 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CUACUACUAC UACTCGAGCA AGATGGGAAC GGACCAAGG

39

10

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39 base pairs

(B) TYPE: nucleic acid

15

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

25

CAUCAUCAUC AUCTCGAGCT ACTCTTCCTT GGGACGGAG

39

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 47 base pairs

(B) TYPE: nucleic acid

30

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: other nucleic acid

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CUACUACUAC UATCTAGACT CGAGACCATG GCTGCTGCTC CAGTGTG

47

(2) INFORMATION FOR SEQ ID NO:28:

45

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40 base pairs

(B) TYPE: nucleic acid

50

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CAUCAUCAUC AUAGGCCTCG AGTTACTGCG CCTTACCCAT

60

40

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CUACUACUA CUAGGATCCA TGGCACCTCC CAACACT

37

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CAUCAUCAU CAUGGTACCT CGAGTTACTT CTTGAAAAAG AC

42

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1219 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid (Edited Contig 2692004)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GCACGCCGAC CGGCGCCGGG AGATCCTGGC AAAGTATCCA GAGATAAAGT CCTTGATGAA 60
ACCTGATCCC AATTGATAT GGATTATAAT TATGATGGTT CTCACCCAGT TGGGTGCATT 120
TTACATAGTA AAAGACTTGG ACTGGAAATG GGTCATATTT GGGGCCTATG CGTTTGGCAG 180
TTGCATTAAC CACTCAATGA CTCTGGCTAT TCATGAGATT GCCCACAATG CTGCCTTTGG 240
CAACTGCAAA GCAATGTGGA ATCGCTGGTT TGGAAATGTTT GCTAATCTTC CTATTGGGAT 300
TCCATATTCA ATTCCTTTA AGAGGTATCA CATGGATCAT CATCGGTACC TTGGAGCTGA 360
TGGCGTCGAT GTAGATATTC CTACCGATTT TGAGGGCTGG TTCTTCTGTA CCGCTTTCAG 420
AAAGTTTATA TGGGTTATTC TTCAGCCTCT CTTTTATGCC TTTCGACCTC TGTTTCATCAA 480

5 CCCCCAACCA ATTACGTATC TGGAAGTTAT CAATACCGTG GCACAGGTCA CTTTGTGACAT 540
 TTTAATTTAT TACTTTTTGG GAATTAAATC CTTAGTCTAC ATGTTGGCAG CATCTTTACT 600
 TGGCCTGGGT TTGCACCCAA TTTCTGGACA TTTTATAGCT GAGCATTACA TGTTCTTAAA 660
 GGGTCATGAA ACTTACTCAT ATTATGGGCC TCTGAATTTA CTTACCTTCA ATGTGGGTTA 720
 10 TCATAATGAA CATCATGATT TCCCCAACAT TCCTGGAAAA AGTCTTCCAC TGGTGAGGAA 780
 AATAGCAGCT GAATACTATG ACAACCTCCC TCACTACAAT TCCTGGATAA AAGTACTGTA 840
 15 TGATTTTGTG ATGGATGATA CAATAAGTCC CTA CTACTCAAGA ATGAAGAGGC ACCAAAAAGG 900
 AGAGATGGTG CTGGAGTAAA TATCATTAGT GCCAAAGGGA TTCTTCTCCA AAACCTTTAGA 960
 TGATAAAATG GAATTTTTGC ATTATTAAAC TTGAGACCAG TGATGCTCAG AAGCTCCCCT 1020
 20 GGCACAATTT CAGAGTAAGA GCTCGGTGAT ACCAAGAAGT GAATCTGGCT TTAAACAGT 1080
 CAGCCTGACT CTGTACTGCT CAGTTTCACT CACAGGAAAC TTGTGACTTG TGTATTATCG 1140
 25 TCATTGAGGA TGTTTCACTC ATGTCTGTCA TTTTATAAGC ATATCATTTA AAAAGCTTCT 1200
 AAAAAGCTAT TTCGCCAGG 1219

(2) INFORMATION FOR SEQ ID NO:32:

30 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 655 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 35 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: other nucleic acid (Edited Contig 2153526)
 40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

45 TTACCTTCTA CGTCCGCTTC TTCCTCACTT ATGTGCCACT ATTGGGGCTG AAAGCTTCCT 60
 GGGCCTTTTC TTCATAGTCA GGTTCCTGGA AAGCAACTGG TTTGTGTGGG TGACACAGAT 120
 GAACCATATT CCCATGCACA TTGATCATGA CCGGAACATG GACTGGGTTT CCACCCAGCT 180
 50 CCAGGCCACA TGCAATGTCC ACAAGTCTGC CTTCAATGAC TGGTTCAGTG GACACCTCAA 240
 CTTCCAGATT GAGCACCATC TTTTCCCAC GATGCCTCGA CACAATTACC ACAAAGTGGC 300
 55 TCCCCTGGTG CAGTCCTTGT GTGCCAAGCA TGGCATAGAG TACCAGTCCA AGCCCCTGCT 360
 GTCAGCCTTC GCCGACATCA TCCACTCACT AAAGGAGTCA GGGCAGCTCT GGCTAGATGC 420
 CTATCTTCAC CAATAACAAC AGCCACCCTG CCCAGTCTGG AAGAAGAGGA GGAAGACTCT 480
 60 GGAGCCAAGG CAGAGGGGAG CTTGAGGGAC AATGCCACTA TAGTTTAATA CTCAGAGGGG 540
 GTTGGGTTTG GGGACATAAA GCCTCTGACT CAAACTCCTC CCTTTTATCT TCTAGCCACA 600

GTTCTAAGAC CCAAAGTGGG GGGTGGACAC AGAAGTCCCT AGGAGGGAAG GAGCT 655

5 (2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 304 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid (Edited Contig 3506132)

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GTCTTTTACT TTGGCAATGG CTGGATTCCT ACCCTCATCA CGGCCTTTGT CCTTGCTACC 60
TCTCAGGCCC AAGCTGGATG GCTGCAACAT GATTATGGCC ACCTGTCTGT CTACAGAAAA 120
20 CCCAAGTGGA ACCACCTTGT CCACAAATTC GTCATTGGCC ACTTAAAGGG TGCCTCTGCC 180
AACTGGTGGA ATCATCGCCA CTTCCAGCAC CACGCCAAGC CTAACATCTT CCACAAGGAT 240
25 CCCGATGTGA ACATGCTGCA CGTGTTTGT CTGGGCGAAT GGCAGCCCAT CGAGTACGGC 300
AAGA 304

30 (2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

- 35 (A) LENGTH: 918 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid (Edited Contig 3854933)

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CAGGGACCTA CCCC GCGCTA CTTACCTGG GACGAGGTGG CCCAGCGCTC AGGGTGCGAG 60
GAGCGGTGGC TAGTGATCGA CCGTAAGGTG TACAACATCA GCGAGTTCAC CCGCCGGCAT 120
45 CCAGGGGGCT CCCGGGTCAT CAGCCACTAC GCCGGGCAGG ATGCCACGGA TCCCTTTGTG 180
GCCTTCCACA TCAACAAGGG CTTGTGAAG AAGTATATGA ACTCTCTCCT GATTGGAGAA 240
50 CTGTCTCCAG AGCAGCCCAG CTTTGAGCCC ACCAAGAATA AAGAGCTGAC AGATGAGTTC 300
CGGGAGCTGC GGGCCACAGT GGAGCGGATG GGGCTCATGA AGGCCAACCA TGTCTTCTTC 360
CTGCTGTACC TGCTGCACAT CTTGCTGCTG GATGGTGAG CCTGGCTCAC CCTTTGGGTC 420
55 TTTGGGACGT CCTTTTGGC CTTCTCCTC TGTGCGGTGC TGCTCAGTGC AGTTCAGGCC 480
CAGGCTGGCT GGCTGCAGCA TGA CTTTGGG CACCTGTCGG TCTTCAGCAC CTCAAAGTGG 540
60 AACCATCTGC TACATCATTT TGTGATTGGC CACCTGAAGG GGGCCCCCGC CAGTTGGTGG 600
AACCACATGC ACTTCCAGCA CCATGCCAAG CCCAACTGCT TCCGCAAAGA CCCAGACATC 660

AACATGCATC CCTTCTTCTT TGCCTTGGGG AAGATCCTCT CTGTGGAGCT TGGGAAACAG 720
 AAGAAAAAAT ATATGCCGTA CAACCACCAG CACARATACT TCTTCCTAAT TGGGCCCCCA 780
 GCCTTGCTGC CTCTCTACTT CCAGTGGTAT ATTTTCTATT TTGTTATCCA GCGAAAGAAG 840
 TGGGTGGACT TGGCCTGGAT CAGCAAACAG GAATACGATG AAGCCGGGCT TCCATTGTCC 900
 ACCGCAAATG CTTCTAAA 918

(2) INFORMATION FOR SEQ ID NO:35:

15 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1686 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 20 (ii) MOLECULE TYPE: other nucleic acid (Edited Contig 2511785)
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:
 25 GCCACTTAAA GGGTGCCTCT GCCAACTGGT GGAATCATCG CCACTTCCAG CACCACGCCA 60
 AGCCTAACAT CTTCCACAAG GATCCCGATG TGAACATGCT GCACGTGTTT GTTCTGGGCG 120
 30 AATGGCAGCC CATCGAGTAC GGCAAGAAGA AGCTGAAATA CCTGCCCTAC AATCACCAGC 180
 ACGAATACTT CTTCTGATT GGGCCGCCGC TGCTCATCCC CATGTATTTT CAGTACCAGA 240
 35 TCATCATGAC CATGATCGTC CATAAGAACT GGGTGGACCT GGCTGGGCC GTCAGCTACT 300
 ACATCCGGTT CTTTCATACC TACATCCCTT TCTACGGCAT CCTGGGAGCC CTCCTTTTCC 360
 TCAACTTCAT CAGGTTCTTG GAGAGCCACT GGTTTGTGTG GGTCACACAG ATGAATCACA 420
 40 TCGTCATGGA GATTGACCAG GAGGCCTACC GTGACTGGTT CAGTAGCCAG CTGACAGCCA 480
 CCTGCAACGT GGAGCAGTCC TTCTTCAACG ACTGGTTCAG TGGACACCTT AACTTCCAGA 540
 45 TTGAGCACCA CCTCTTCCCC ACCATGCCCC GGCACAACTT ACACAAGATC GCCCCGCTGG 600
 TGAAGTCTCT ATGTGCCAAG CATGGCATTG AATACCAGGA GAAGCCGCTA CTGAGGGCCC 660
 TGCTGGACAT CATCAGGTCC CTGAAGAAGT CTGGGAAGCT GTGGCTGGAC GCCTACCTTC 720
 50 ACAAATGAAG CCACAGCCCC CGGGACACCG TGGGGAAGGG GTGCAGGTGG GGTGATGGCC 780
 AGAGGAATGA TGGGCTTTTG TTCTGAGGGG TGTCCGAGAG GCTGGTGTAT GCACTGCTCA 840
 CGGACCCCAT GTTGATCTT TCTCCCTTTC TCCTCTCCTT TTTCTCTTCA CATCTCCCCC 900
 55 ATAGCACCCCT GCCCTCATGG GACCTGCCCT CCCTCAGCCG TCAGCCATCA GCCATGGCCC 960
 TCCCAGTGCC TCCTAGCCCC TTCTTCCAAG GAGCAGAGAG GTGGCCACCG GGGGTGGCTC 1020
 60 TGTCTACCT CCACTCTCTG CCCCTAAAGA TGGGAGGAGA CCAGCGGTCC ATGGGTCTGG 1080
 CCTGTGAGTC TCCCCTTGCA GCCTGGTCAC TAGGCATCAC CCCCCTTTG GTTCTTCAGA 1140

5 TGCTCTTGGG GTTCATAGGG GCAGGTCCTA GTCGGGCAGG GCCCCTGACC CTCCCGGCCT 1200
 GGCTTCACTC TCCCTGACGG CTGCCATTGG TCCACCCTTT CATAGAGAGG CCTGCTTTGT 1260
 TACAAAGCTC GGGTCTCCCT CCTGCAGCTC GGTAAAGTAC CCGAGGCCTC TCTTAAGATG 1320
 TCCAGGGCCC CAGGCCCCGCG GGCACAGCCA GCCCAAACCT TGGGCCCTGG AAGAGTCCTC 1380
 10 CACCCCATCA CTAGAGTGCT CTGACCCTGG GCTTTCACGG GCCCCATTCC ACCGCCTCCC 1440
 CAACTTGAGC CTGTGACCTT GGGACCAAAG GGGGAGTCCC TCGTCTCTTG TGA CTGAGCA 1500
 GAGGCAGTGG CCACGTTTCA GAGGGGGCCG GCTGGCCTGG AGGCTCAGCC CACCCTCCAG 1560
 15 CTTTTCCTCA GGGTGTCTTG AGGTCCAAGA TTCTGGAGCA ATCTGACCCT TCTCAAAGG 1620
 CTCTGTTATC AGCTGGGCAG TGCCAGCCAA TCCCTGGCCA TTTGGCCCCA GGGGACGTGG 1680
 20 GCCCTG 1686

(2) INFORMATION FOR SEQ ID NO:36:

25 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1843 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 30 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: other nucleic acid (Contig 2535)
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

35 GTCTTTTACT TTGGCAATGG CTGGATTCTT ACCCTCATCA CGGCCTTTGT CCTTGCTACC 60
 TCTCAGGCCC AAGCTGGATG GCTGCAACAT GATTATGGCC ACCTGTCTGT CTACAGAAAA 120
 40 CCCAAGTGGA ACCACCTTGT CCACAAATTC GTCATTGGCC ACTTAAAGGG TGCCTCTGCC 180
 AACTGGTGGA ATCATCGCCA CTTCAGCAC CACGCCAAGC CTAACATCTT CCACAAGGAT 240
 45 CCCGATGTGA ACATGCTGCA CGTGTGTTT CTGGGCGAAT GGCAGCCCAT CGAGTACGGC 300
 AAGAAGAAGC TGAAATACCT GCCCTACAAT CACCAGCACG AATACTTCTT CCTGATTGGG 360
 CCGCCGCTGC TCATCCCCAT GTATTTCCAG TACCAGATCA TCATGACCAT GATCGTCCAT 420
 50 AAGAACTGGG TGGACCTGGC CTGGGCCGTC AGCTACTACA TCCGGTTCTT CATCACCTAC 480
 ATCCCTTTCT ACGGCATCCT GGGAGCCCTC CTTTTCCTCA ACTTCATCAG GTTCCTGGAG 540
 AGCCACTGGT TTGTGTGGGT CACACAGATG AATCACATCG TCATGGAGAT TGACCAGGAG 600
 55 GCCTACCGTG ACTGGTTCAG TAGCCAGCTG ACAGCCACCT GCAACGTGGA GCAGTCCTTC 660
 TTCAACGACT GGTTCAGTGG ACACCTTAAC TTCCAGATTG AGCACCACCT CTTCCCCACC 720
 60 ATGCCCCGGC ACAACTTACA CAAGATCGCC CCGCTGGTGA AGTCTCTATG TGCCAAGCAT 780
 GGCATTGAAT ACCAGGAGAA GCCGCTACTG AGGGCCCTGC TGGACATCAT CAGGTCCCTG 840

AAGAAGTCTG GGAAGCTGTG GCTGGACGCC TACCTTCACA AATGAAGCCA CAGCCCCCGG 900
 5 GACACCGTGG GGAAGGGGTG CAGGTGGGGT GATGGCCAGA GGAATGATGG GCTTTTGTTC 960
 TGAGGGGTGT CCGAGAGGCT GGTGTATGCA CTGCTCACGG ACCCATGTT GGATCTTTCT 1020
 CCCTTTCTCC TCTCCTTTTT CTCTTCACAT CTCCCCATA GCACCCTGCC CTCATGGGAC 1080
 10 CTGCCCTCCC TCAGCCGTCA GCCATCAGCC ATGGCCCTCC CAGTGCCTCC TAGCCCCTTC 1140
 TTCCAAGGAG CAGAGAGGTG GCCACCGGGG GTGGCTCTGT CCTACCTCCA CTCTCTGCCC 1200
 15 CTAAAGATGG GAGGAGACCA GCGGTCCATG GGTCTGGCCT GTGAGTCTCC CTTGCGAGCC 1260
 TGGTCACTAG GCATACCCC CGCTTTGGTT CTTCAGATGC TCTTGGGGTT CATAGGGGCA 1320
 GGTCCCTAGTC GGGCAGGGCC CCTGACCCTC CCGGCCTGGC TTCCTCTCC CTGACGGCTG 1380
 20 CCATTGGTCC ACCCTTTCAT AGAGAGGCCT GCTTTGTTAC AAAGCTCGGG TCTCCCTCCT 1440
 GCAGCTCGGT TAAGTACCCG AGGCCTCTCT TAAGATGTCC AGGGCCCCAG GCCCGCGGGC 1500
 ACAGCCAGCC CAAACCTTGG GCCCTGGAAG AGTCCTCCAC CCCATCACTA GAGTGCTCTG 1560
 25 ACCCTGGGCT TTCACGGGCC CCATTCCACC GCCTCCCCAA CTTGAGCCTG TGACCTTGGG 1620
 ACCAAAGGGG GAGTCCCTCG TCTCTTGTGA CTCAGCAGAG GCAGTGGCCA CGTTCAGGGA 1680
 30 GGGGCCGGCT GGCCTGGAGG CTCAGCCCAC CCTCCAGCTT TTCCTCAGGG TGTCTGAGG 1740
 TCCAAGATTC TGGAGCAATC TGACCCTTCT CCAAAGGCTC TGTTATCAGC TGGGCAGTGC 1800
 CAGCCAATCC CTGGCCATTT GGCCCCAGGG GACGTGGGCC CTG 1843
 35

-(2) INFORMATION FOR SEQ ID NO:37:

- 40 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 2257 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 45 (ii) MOLECULE TYPE: other nucleic acid (Edited Contig 253538a)
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

50 CAGGGACCTA CCCCGCGCTA CTTACCTGG GACGAGGTGG CCCAGCGCTC AGGGTGCGAG 60
 GAGCGGTGGC TAGTGATCGA CCGTAAGGTG TACAACATCA GCGAGTTCAC CCGCCGGCAT 120
 CCAGGGGGCT CCCGGGTCAT CAGCCACTAC GCCGGGCAGG ATGCCACGGA TCCCTTTGTG 180
 55 GCCTTCCACA TCAACAAGGG CCTTGTGAAG AAGTATATGA ACTCTCTCCT GATTGGAGAA 240
 CTGTCTCCAG AGCAGCCCAG CTTTGAGCCC ACCAAGAATA AAGAGCTGAC AGATGAGTTC 300
 CGGGAGCTGC GGGCCACAGT GGAGCGGATG GGGCTCATGA AGGCCAACCA TGTCTTCTTC 360
 60 CTGCTGTACC TGCTGCACAT CTTGCTGCTG GATGGTGCAG CCTGGCTCAC CTTTGGGTG 420

	TTTGGGACGT CCTTTTGGCC CTTCTCCTC TGTGCGGTGC TGCTCAGTGC AGTTCAGCAG	480
	GCCCAAGCTG GATGGCTGCA ACATGATTAT GGCCACCTGT CTGTCTACAG AAAACCCAAG	540
5	TGGAACCACC TTGTCCACAA ATTCGTCATT GGCCACTTAA AGGGTGCCTC TGCCAACCTGG	600
	TGGAATCATC GCCACTTCCA GCACCACGCC AAGCCTAACA TCTTCCACAA GGATCCCGAT	660
10	GTGAACATGC TGCACGTGTT TGTCTGGGC GAATGGCAGC CCATCGAGTA CGGCAAGAAG	720
	AAGCTGAAAT ACCTGCCCTA CAATCACCAG CACGAATACT TCTTCTGAT TGGGCCGCCG	780
	CTGCTCATCC CCATGTATTT CCAGTACCAG ATCATCATGA CCATGATCGT CCATAAGAAC	840
15	TGGGTGGACC TGGCCTGGGC CGTCAGCTAC TACATCCGGT TCTTCATCAC CTACATCCCT	900
	TTCTACGGCA TCCTGGGAGC CCTCCTTTTC CTCAACTTCA TCAGGTTCTT GGAGAGCCAC	960
20	TGGTTTGTGT GGGTCACACA GATGAATCAC ATCGTCATGG AGATTGACCA GGAGGCCTAC	1020
	CGTGACTGGT TCAGTAGCCA GCTGACAGCC ACCTGCAACG TGGAGCAGTC CTTCTTCAAC	1080
	GACTGGTTCA GTGGACACCT TAACTTCCAG ATTGAGCACC ACCTCTTCCC CACCATGCCC	1140
25	CGGCACAAC TACACAAGAT CGCCCCGCTG GTGAAGTCTC TATGTGCCAA GCATGGCATT	1200
	GAATACCAGG AGAAGCCGCT ACTGAGGGCC CTGCTGGACA TCATCAGGTC CCTGAAGAAG	1260
30	TCTGGGAAGC TGTGGCTGGA CGCCTACCTT CACAAATGAA GCCACAGCCC CCGGGACACC	1320
	GTGGGGAAGG GGTGCAGGTG GGGTGATGGC CAGAGGAATG ATGGGCTTTT GTTCTGAGGG	1380
	GTGTCCGAGA GGCTGGTGTA TGCACTGCTC ACGGACCCCA TGTTGGATCT TTCTCCCTTT	1440
35	CTCCTCTCCT TTTTCTCTT ACATCTCCCC CATAGCACCC TGCCCTCATG GGACCTGCCC	1500
	TCCCTCAGCC GTCAGCCATC AGCCATGGCC CTCCCAGTGC CTCCTAGCCC CTTCTTCCAA	1560
40	GGAGCAGAGA GGTGGCCACC GGGGGTGGCT CTGTCCTACC TCCACTCTCT GCCCCTAAAG	1620
	ATGGGAGGAG ACCAGCGGTC CATGGGTCTG GCCTGTGAGT CTCCCCTTGC AGCCTGGTCA	1680
	CTAGGCATCA CCCCCGCTTT GGTCTTTCAG ATGCTCTTGG GGTTCATAGG GGCAGGTCTT	1740
45	AGTCGGGCAG GGCCCCTGAC CCTCCCGGCC TGGCTTCACT CTCCCTGACG GGTGCCATTG	1800
	GTCCACCCTT TCATAGAGAG GCCTGCTTTG TTACAAAGCT CGGGTCTCCC TCCTGCAGCT	1860
50	CGGTTAAGTA CCCGAGGCCT CTCTTAAGAT GTCCAGGGCC CCAGGCCCGC GGGCACAGCC	1920
	AGCCCAAACC TTGGGCCCTG GAAGAGTCCT CCACCCCATC ACTAGAGTGC TCTGACCCTG	1980
	GGCTTTCACG GGCCCCATTC CACCGCTCC CCAACTTGAG CCTGTGACCT TGGGACCAAA	2040
55	GGGGGAGTCC CTCGTCTCTT GTGACTCAGC AGAGGCAGTG GCCACGTTCA GGGAGGGGCC	2100
	GGCTGGCCTG GAGGCTCAGC CCACCCTCCA GCTTTTCTC AGGGTGTCTT GAGGTCCAAG	2160
60	ATTCTGGAGC AATCTGACCC TTCTCCAAAG GCTCTGTTAT CAGCTGGGCA GTGCCAGCCA	2220
	ATCCCTGGCC ATTTGGCCCC AGGGGACGTG GGCCCTG	2257

(2) INFORMATION FOR SEQ ID NO:38:

- (i) SEQUENCE CHARACTERISTICS:
- 5 (A) LENGTH: 411 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- 10 (ii) MOLECULE TYPE: amino acid (Translation of Contig 2692004)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

15	His	Ala	Asp	Arg	Arg	Arg	Glu	Ile	Leu	Ala	Lys	Tyr	Pro	Glu	Ile	1	5	10	15
	Lys	Ser	Leu	Met	Lys	Pro	Asp	Pro	Asn	Leu	Ile	Trp	Ile	Ile	Ile	20	25	30	
	Met	Met	Val	Leu	Thr	Gln	Leu	Gly	Ala	Phe	Tyr	Ile	Val	Lys	Asp	35	40	45	
20	Leu	Asp	Trp	Lys	Trp	Val	Ile	Phe	Gly	Ala	Tyr	Ala	Phe	Gly	Ser	50	55	60	
	Cys	Ile	Asn	His	Ser	Met	Thr	Leu	Ala	Ile	His	Glu	Ile	Ala	His	65	70	75	
25	Asn	Ala	Ala	Phe	Gly	Asn	Cys	Lys	Ala	Met	Trp	Asn	Arg	Trp	Phe	80	85	90	
	Gly	Met	Phe	Ala	Asn	Leu	Pro	Ile	Gly	Ile	Pro	Tyr	Ser	Ile	Ser	95	100	105	
30	Phe	Lys	Arg	Tyr	His	Met	Asp	His	His	Arg	Tyr	Leu	Gly	Ala	Asp	110	115	120	
	Gly	Val	Asp	Val	Asp	Ile	Pro	Thr	Asp	Phe	Glu	Gly	Trp	Phe	Phe	125	130	135	
	Cys	Thr	Ala	Phe	Arg	Lys	Phe	Ile	Trp	Val	Ile	Leu	Gln	Pro	Leu	140	145	150	
35	Phe	Tyr	Ala	Phe	Arg	Pro	Leu	Phe	Ile	Asn	Pro	Lys	Pro	Ile	Thr	155	160	165	
	Tyr	Leu	Glu	Val	Ile	Asn	Thr	Val	Ala	Gln	Val	Thr	Phe	Asp	Ile	170	175	180	
40	Leu	Ile	Tyr	Tyr	Phe	Leu	Gly	Ile	Lys	Ser	Leu	Val	Tyr	Met	Leu	185	190	195	
	Ala	Ala	Ser	Leu	Gly	Leu	Gly	Leu	His	Pro	Ile	Ser	Gly	His		200	205	210	
	Phe	Ile	Ala	Glu	His	Tyr	Met	Phe	Leu	Lys	Gly	His	Glu	Thr	Tyr	215	220	225	
45	Ser	Tyr	Tyr	Gly	Pro	Leu	Asn	Leu	Leu	Thr	Phe	Asn	Val	Gly	Tyr	230	235	240	
	His	Asn	Glu	His	His	Asp	Phe	Pro	Asn	Ile	Pro	Gly	Lys	Ser	Leu	245	250	255	
	Pro	Leu	Val	Arg	Lys	Ile	Ala	Ala	Glu	Tyr	Tyr	Asp	Asn	Leu	Pro	260	265	270	
50	His	Tyr	Asn	Ser	Trp	Ile	Lys	Val	Leu	Tyr	Asp	Phe	Val	Met	Asp	275	280	285	
	Asp	Thr	Ile	Ser	Pro	Tyr	Ser	Arg	Met	Lys	Arg	His	Gln	Lys	Gly	290	295	300	
55	Glu	Met	Val	Leu	Glu	***	Ile	Ser	Leu	Val	Pro	Lys	Gly	Phe	Phe	305	310	315	
	Ser	Lys	Thr	Leu	Asp	Asp	Lys	Met	Glu	Phe	Leu	His	Tyr	***	Thr	320	325	330	
	***	Asp	Gln	***	Cys	Ser	Glu	Ala	Pro	Leu	Ala	Gln	Phe	Gln	Ser	335	340	345	
60	Lys	Ser	Ser	Val	Ile	Pro	Arg	Ser	Glu	Ser	Gly	Phe	***	Thr	Val	350	355	360	

Ser Leu Thr Leu Tyr Cys Ser Val Ser Leu Thr Gly Asn Leu ***
 365 370 375
 Leu Val Tyr Tyr Arg His *** Gly Cys Phe Thr His Val Cys His
 380 385 390
 5 Phe Ile Ser Ile Ser Phe Lys Lys Leu Leu Lys Ser Tyr Phe Ala
 400 405 410
 Arg

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 218 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid (Translation of Contig 2153526)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

Tyr Leu Leu Arg Pro Leu Leu Pro His Leu Cys Ala Thr Ile Gly
 1 5 10 15
 Ala Glu Ser Phe Leu Gly Leu Phe Phe Ile Val Arg Phe Leu Glu
 20 25 30
 Ser Asn Trp Phe Val Trp Val Thr Gln Met Asn His Ile Pro Met
 35 40 45
 His Ile Asp His Asp Arg Asn Met Asp Trp Val Ser Thr Gln Leu
 50 55 60
 30 Gln Ala Thr Cys Asn Val His Lys Ser Ala Phe Asn Asp Trp Phe
 65 70 75
 Ser Gly His Leu Asn Phe Gln Ile Glu His His Leu Phe Pro Thr
 80 85 90
 Met Pro Arg His Asn Tyr His Lys Val Ala Pro Leu Val Gln Ser
 95 100 105
 35 Leu Cys Ala Lys His Gly Ile Glu Tyr Gln Ser Lys Pro Leu Leu
 110 115 120
 Ser Ala Phe Ala Asp Ile Ile His Ser Leu Lys Glu Ser Gly Gln
 125 130 135
 40 Leu Trp Leu Asp Ala Tyr Leu His Gln *** Gln Gln Pro Pro Cys
 140 145 150
 Pro Val Trp Lys Lys Arg Arg Lys Thr Leu Glu Pro Arg Gln Arg
 155 160 165
 Gly Ala *** Gly Thr Met Pro Leu *** Phe Asn Thr Gln Arg Gly
 170 175 180
 45 Leu Gly Leu Gly Thr *** Ser Leu *** Leu Lys Leu Leu Pro Phe
 185 190 195
 Ile Phe *** Pro Gln Phe *** Asp Pro Lys Trp Gly Val Asp Thr
 200 205 210
 50 Glu Val Pro Arg Arg Glu Gly Ala
 215

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 71 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid (Translation of Contig 3506132)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

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Val Phe Tyr Phe Gly Asn Gly Trp Ile Pro Thr Leu Ile Thr Ala
1          5          10          15
Phe Val Leu Ala Thr Ser Gln Ala Gln Ala Gly Trp Leu Gln His
20          25          30
Asp Tyr Gly His Leu Ser Val Tyr Arg Lys Pro Lys Trp Asn His
35          40          45
Leu Val His Lys Phe Val Ile Gly His Leu Lys Gly Ala Ser Ala
50          55          60
Asn Trp Trp Asn His Arg His Phe Gln His His Ala Lys Pro Asn
65          70          75
Leu Gly Glu Trp Gln Pro Ile Glu Tyr Gly Lys Xxx
80          85

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(2) INFORMATION FOR SEQ ID NO:41:

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(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 306 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: amino acid (Translation of Contig 3854933)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

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Gln Gly Pro Thr Pro Arg Tyr Phe Thr Trp Asp Glu Val Ala Gln
1          5          10          15
Arg Ser Gly Cys Glu Glu Arg Trp Leu Val Ile Asp Arg Lys Val
20          25          30
Tyr Asn Ile Ser Glu Phe Thr Arg Arg His Pro Gly Gly Ser Arg
35          40          45
Val Ile Ser His Tyr Ala Gly Gln Asp Ala Thr Asp Pro Phe Val
50          55          60
Ala Phe His Ile Asn Lys Gly Leu Val Lys Lys Tyr Met Asn Ser
65          70          75
Leu Leu Ile Gly Glu Leu Ser Pro Glu Gln Pro Ser Phe Glu Pro
80          85          90
Thr Lys Asn Lys Glu Leu Thr Asp Glu Phe Arg Glu Leu Arg Ala
95          100          105
Thr Val Glu Arg Met Gly Leu Met Lys Ala Asn His Val Phe Phe
110          115          120
Leu Leu Tyr Leu Leu His Ile Leu Leu Leu Asp Gly Ala Ala Trp
125          130          135
Leu Thr Leu Trp Val Phe Gly Thr Ser Phe Leu Pro Phe Leu Leu
140          145          150
Cys Ala Val Leu Leu Ser Ala Val Gln Ala Gln Ala Gly Trp Leu
155          160          165
Gln His Asp Phe Gly His Leu Ser Val Phe Ser Thr Ser Lys Trp
170          175          180
Asn His Leu Leu His His Phe Val Ile Gly His Leu Lys Gly Ala
185          190          195
Pro Ala Ser Trp Trp Asn His Met His Phe Gln His His Ala Lys
200          205          210

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5 Pro Asn Cys Phe Arg Lys Asp Pro Asp Ile Asn Met His Pro Phe
 215 220 225
 Phe Phe Ala Leu Gly Lys Ile Leu Ser Val Glu Leu Gly Lys Gln
 230 235 240
 10 Lys Lys Lys Tyr Met Pro Tyr Asn His Gln His Xxx Tyr Phe Phe
 245 250 255
 Leu Ile Gly Pro Pro Ala Leu Leu Pro Leu Tyr Phe Gln Trp Tyr
 260 265 270
 15 Ile Phe Tyr Phe Val Ile Gln Arg Lys Lys Trp Val Asp Leu Ala
 275 280 285
 Trp Ile Ser Lys Gln Glu Tyr Asp Glu Ala Gly Leu Pro Leu Ser
 290 295 300
 Thr Ala Asn Ala Ser Lys
 305

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 566 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid (Translation of Contig 2511785)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

30 His Leu Lys Gly Ala Ser Ala Asn Trp Trp Asn His Arg His Phe
 1 5 10 15
 Gln His His Ala Lys Pro Asn Ile Phe His Lys Asp Pro Asp Val
 20 25 30
 35 Asn Met Leu His Val Phe Val Leu Gly Glu Trp Gln Pro Ile Glu
 35 40 45
 Tyr Gly Lys Lys Lys Leu Lys Tyr Leu Pro Tyr Asn His Gln His
 50 55 60
 Glu Tyr Phe Phe Leu Ile Gly Pro Pro Leu Leu Ile Pro Met Tyr
 65 70 75
 40 Phe Gln Tyr Gln Ile Ile Met Thr Met Ile Val His Lys Asn Trp
 80 85 90
 Val Asp Leu Ala Trp Ala Val Ser Tyr Tyr Ile Arg Phe Phe Ile
 95 100 105
 45 Thr Tyr Ile Pro Phe Tyr Gly Ile Leu Gly Ala Leu Leu Phe Leu
 110 115 120
 Asn Phe Ile Arg Phe Leu Glu Ser His Trp Phe Val Trp Val Thr
 125 130 135
 Gln Met Asn His Ile Val Met Glu Ile Asp Gln Glu Ala Tyr Arg
 140 145 150
 50 Asp Trp Phe Ser Ser Gln Leu Thr Ala Thr Cys Asn Val Glu Gln
 155 160 165
 Ser Phe Phe Asn Asp Trp Phe Ser Gly His Leu Asn Phe Gln Ile
 170 175 180
 55 Glu His His Leu Phe Pro Thr Met Pro Arg His Asn Leu His Lys
 185 190 195
 Ile Ala Pro Leu Val Lys Ser Leu Cys Ala Lys His Gly Ile Glu
 200 205 210
 Tyr Gln Glu Lys Pro Leu Leu Arg Ala Leu Leu Asp Ile Ile Arg
 215 220 225
 60 Ser Leu Lys Lys Ser Gly Lys Leu Trp Leu Asp Ala Tyr Leu His
 230 235 240
 Lys *** Ser His Ser Pro Arg Asp Thr Val Gly Lys Gly Cys Arg

		245		250		255
	Trp Gly Asp Gly	Gln Arg Asn Asp Gly	Leu Leu Phe *** Gly	Val		
		260		265		270
5	Ser Glu Arg Leu	Val Tyr Ala Leu Leu	Thr Asp Pro Met Leu	Asp		
		275		280		285
	Leu Ser Pro Phe	Leu Leu Ser Phe Phe	Ser Ser His Leu Pro	His		
		290		295		300
	Ser Thr Leu Pro	Ser Trp Asp Leu Pro	Ser Leu Ser Arg Gln	Pro		
		305		310		315
10	Ser Ala Met Ala	Leu Pro Val Pro Pro	Ser Pro Phe Phe Gln	Gly		
		320		325		330
	Ala Glu Arg Trp	Pro Pro Gly Val Ala	Leu Ser Tyr Leu His	Ser		
		335		340		345
15	Leu Pro Leu Lys	Met Gly Gly Asp Gln	Arg Ser Met Gly Leu	Ala		
		350		355		360
	Cys Glu Ser Pro	Leu Ala Ala Trp Ser	Leu Gly Ile Thr Pro	Ala		
		365		370		375
	Leu Val Leu Gln	Met Leu Leu Gly Phe	Ile Gly Ala Gly Pro	Ser		
		380		385		390
20	Arg Ala Gly Pro	Leu Thr Leu Pro Ala	Trp Leu His Ser Pro	***		
		400		405		410
	Arg Leu Pro Leu	Val His Pro Phe Ile	Glu Arg Pro Ala Leu	Leu		
		415		420		425
25	Gln Ser Ser Gly	Leu Pro Pro Ala Ala	Arg Leu Ser Thr Arg	Gly		
		430		435		440
	Leu Ser *** Asp	Val Gln Gly Pro Arg	Pro Ala Gly Thr Ala	Ser		
		445		450		455
	Pro Asn Leu Gly	Pro Trp Lys Ser Pro	Pro Pro His His ***	Ser		
		460		465		470
30	Ala Leu Thr Leu	Gly Phe His Gly Pro	His Ser Thr Ala Ser	Pro		
		475		480		485
	Thr *** Ala Cys	Asp Leu Gly Thr Lys	Gly Gly Val Pro Arg	Leu		
		490		495		500
35	Leu *** Leu Ser	Arg Gly Ser Gly His	Val Gln Gly Gly Ala	Gly		
		505		510		515
	Trp Pro Gly Gly	Ser Ala His Pro Pro	Ala Phe Pro Gln Gly	Val		
		520		525		530
	Leu Arg Ser Lys	Ile Leu Glu Gln Ser	Asp Pro Ser Pro Lys	Ala		
		535		540		545
40	Leu Leu Ser Ala	Gly Gln Cys Gln Pro	Ile Pro Gly His Leu	Ala		
		550		555		560
	Pro Gly Asp Val	Gly Pro Xxx				
		565				

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(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 619 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: amino acid (Translation of Contig 2535)

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

60

Val Phe Tyr Phe Gly Asn Gly Trp Ile Pro Thr Leu Ile Thr Ala	
1	5 10 15
Phe Val Leu Ala Thr Ser Gln Ala Gln Ala Gly Trp Leu Gln His	

					20					25					30
	Asp	Tyr	Gly	His	Leu	Ser	Val	Tyr	Arg	Lys	Pro	Lys	Trp	Asn	His
					35					40					45
5	Leu	Val	His	Lys	Phe	Val	Ile	Gly	His	Leu	Lys	Gly	Ala	Ser	Ala
					50					55					60
	Asn	Trp	Trp	Asn	His	Arg	His	Phe	Gln	His	His	Ala	Lys	Pro	Asn
					65					70					75
	Ile	Phe	His	Lys	Asp	Pro	Asp	Val	Asn	Met	Leu	His	Val	Phe	Val
					80					85					90
10	Leu	Gly	Glu	Trp	Gln	Pro	Ile	Glu	Tyr	Gly	Lys	Lys	Lys	Leu	Lys
					95					100					105
	Tyr	Leu	Pro	Tyr	Asn	His	Gln	His	Glu	Tyr	Phe	Phe	Leu	Ile	Gly
					110					115					120
	Pro	Pro	Leu	Leu	Ile	Pro	Met	Tyr	Phe	Gln	Tyr	Gln	Ile	Ile	Met
15					125					130					135
	Thr	Met	Ile	Val	His	Lys	Asn	Trp	Val	Asp	Leu	Ala	Trp	Ala	Val
					140					145					150
	Ser	Tyr	Tyr	Ile	Arg	Phe	Phe	Ile	Thr	Tyr	Ile	Pro	Phe	Tyr	Gly
					155					160					165
20	Ile	Leu	Gly	Ala	Leu	Leu	Phe	Leu	Asn	Phe	Ile	Arg	Phe	Leu	Glu
					170					175					180
	Ser	His	Trp	Phe	Val	Trp	Val	Thr	Gln	Met	Asn	His	Ile	Val	Met
					185					190					195
	Glu	Ile	Asp	Gln	Glu	Ala	Tyr	Arg	Asp	Trp	Phe	Ser	Ser	Gln	Leu
25					200					205					210
	Thr	Ala	Thr	Cys	Asn	Val	Glu	Gln	Ser	Phe	Phe	Asn	Asp	Trp	Phe
					215					220					225
	Ser	Gly	His	Leu	Asn	Phe	Gln	Ile	Glu	His	His	Leu	Phe	Pro	Thr
					230					235					240
30	Met	Pro	Arg	His	Asn	Leu	His	Lys	Ile	Ala	Pro	Leu	Val	Lys	Ser
					245					250					255
	Leu	Cys	Ala	Lys	His	Gly	Ile	Glu	Tyr	Gln	Glu	Lys	Pro	Leu	Leu
					260					265					270
	Arg	Ala	Leu	Leu	Asp	Ile	Ile	Arg	Ser	Leu	Lys	Lys	Ser	Gly	Lys
35					275					280					285
	Leu	Trp	Leu	Asp	Ala	Tyr	Leu	His	Lys	***	Ser	His	Ser	Pro	Arg
					290					295					300
	Asp	Thr	Val	Gly	Lys	Gly	Cys	Arg	Trp	Gly	Asp	Gly	Gln	Arg	Asn
					305					310					315
40	Asp	Gly	Leu	Leu	Phe	***	Gly	Val	Ser	Glu	Arg	Leu	Val	Tyr	Ala
					320					325					330
	Leu	Leu	Thr	Asp	Pro	Met	Leu	Asp	Leu	Ser	Pro	Phe	Leu	Leu	Ser
					335					340					345
	Phe	Phe	Ser	Ser	His	Leu	Pro	His	Ser	Thr	Leu	Pro	Ser	Trp	Asp
45					350					355					360
	Leu	Pro	Ser	Leu	Ser	Arg	Gln	Pro	Ser	Ala	Met	Ala	Leu	Pro	Val
					365					370					375
	Pro	Pro	Ser	Pro	Phe	Phe	Gln	Gly	Ala	Glu	Arg	Trp	Pro	Pro	Gly
					380					385					390
50	Val	Ala	Leu	Ser	Tyr	Leu	His	Ser	Leu	Pro	Leu	Lys	Met	Gly	Gly
					400					405					410
	Asp	Gln	Arg	Ser	Met	Gly	Leu	Ala	Cys	Glu	Ser	Pro	Leu	Ala	Ala
					415					420					425
	Trp	Ser	Leu	Gly	Ile	Thr	Pro	Ala	Leu	Val	Leu	Gln	Met	Leu	Leu
55					430					435					440
	Gly	Phe	Ile	Gly	Ala	Gly	Pro	Ser	Arg	Ala	Gly	Pro	Leu	Thr	Leu
					445					450					455
	Pro	Ala	Trp	Leu	His	Ser	Pro	***	Arg	Leu	Pro	Leu	Val	His	Pro
					460					465					470
60	Phe	Ile	Glu	Arg	Pro	Ala	Leu	Leu	Gln	Ser	Ser	Gly	Leu	Pro	Pro
					475					480					485
	Ala	Ala	Arg	Leu	Ser	Thr	Arg	Gly	Leu	Ser	***	Asp	Val	Gln	Gly

		490		495		500
	Pro Arg Pro Ala	Gly Thr Ala Ser Pro	Asn Leu Gly Pro Trp	Lys		
		505		510		515
5	Ser Pro Pro Pro	His His *** Ser Ala	Leu Thr Leu Gly Phe	His		
		520		525		530
	Gly Pro His Ser	Thr Ala Ser Pro Thr	*** Ala Cys Asp Leu	Gly		
		535		540		545
	Thr Lys Gly Gly	Val Pro Arg Leu Leu	*** Leu Ser Arg Gly	Ser		
		550		555		560
10	Gly His Val Gln	Gly Gly Ala Gly Trp	Pro Gly Gly Ser Ala	His		
		565		570		575
	Pro Pro Ala Phe	Pro Gln Gly Val Leu	Arg Ser Lys Ile Leu	Glu		
		580		585		590
	Gln Ser Asp Pro	Ser Pro Lys Ala Leu	Leu Ser Ala Gly Gln	Cys		
15		595		600		605
	Gln Pro Ile Pro	Gly His Leu Ala Pro	Gly Asp Val Gly Pro	Xxx		
		610		615		620

20

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 757 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: amino acid (Translation of Contig 253538a)

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

35	Gln Gly Pro Thr Pro Arg Tyr Phe Thr Trp Asp Glu Val Ala Gln	1	5	10	15
	Arg Ser Gly Cys Glu Glu Arg Trp Leu Val Ile Asp Arg Lys Val	20	25	30	
	Tyr Asn Ile Ser Glu Phe Thr Arg Arg His Pro Gly Gly Ser Arg	35	40	45	
40	Val Ile Ser His Tyr Ala Gly Gln Asp Ala Thr Asp Pro Phe Val	50	55	60	
	Ala Phe His Ile Asn Lys Gly Leu Val Lys Lys Tyr Met Asn Ser	65	70	75	
45	Leu Leu Ile Gly Glu Leu Ser Pro Glu Gln Pro Ser Phe Glu Pro	80	85	90	
	Thr Lys Asn Lys Glu Leu Thr Asp Glu Phe Arg Glu Leu Arg Ala	95	100	105	
	Thr Val Glu Arg Met Gly Leu Met Lys Ala Asn His Val Phe Phe	110	115	120	
50	Leu Leu Tyr Leu Leu His Ile Leu Leu Asp Gly Ala Ala Trp	125	130	135	
	Leu Thr Leu Trp Val Phe Gly Thr Ser Phe Leu Pro Phe Leu Leu	140	145	150	
55	Cys Ala Val Leu Leu Ser Ala Val Gln Gln Ala Gln Ala Gly Trp	155	160	165	
	Leu Gln His Asp Tyr Gly His Leu Ser Val Tyr Arg Lys Pro Lys	170	175	180	
	Trp Asn His Leu Val His Lys Phe Val Ile Gly His Leu Lys Gly	185	190	195	
60	Ala Ser Ala Asn Trp Trp Asn His Arg His Phe Gln His His Ala	200	205	210	
	Lys Pro Asn Ile Phe His Lys Asp Pro Asp Val Asn Met Leu His				

		215		220		225
	Val Phe Val Leu	Gly Glu Trp Gln Pro	Ile Glu Tyr Gly Lys	Lys		
		230		235		240
5	Lys Leu Lys Tyr	Leu Pro Tyr Asn His	Gln His Glu Tyr Phe	Phe		
		245		250		255
	Leu Ile Gly Pro	Pro Leu Leu Ile Pro	Met Tyr Phe Gln Tyr	Gln		
		260		265		270
	Ile Ile Met Thr	Met Ile Val His Lys	Asn Trp Val Asp Leu	Ala		
		275		280		285
10	Trp Ala Val Ser	Tyr Tyr Ile Arg Phe	Phe Ile Thr Tyr Ile	Pro		
		290		295		300
	Phe Tyr Gly Ile	Leu Gly Ala Leu Leu	Phe Leu Asn Phe Ile	Arg		
		305		310		315
	Phe Leu Glu Ser	His Trp Phe Val Trp	Val Thr Gln Met Asn	His		
15		320		325		330
	Ile Val Met Glu	Ile Asp Gln Glu Ala	Tyr Arg Asp Trp Phe	Ser		
		335		340		345
	Ser Gln Leu Thr	Ala Thr Cys Asn Val	Glu Gln Ser Phe Phe	Asn		
		350		355		360
20	Asp Trp Phe Ser	Gly His Leu Asn Phe	Gln Ile Glu His His	Leu		
		365		370		375
	Phe Pro Thr Met	Pro Arg His Asn Leu	His Lys Ile Ala Pro	Leu		
		380		385		390
	Val Lys Ser Leu	Cys Ala Lys His Gly	Ile Glu Tyr Gln Glu	Lys		
25		400		405		410
	Pro Leu Leu Arg	Ala Leu Leu Asp Ile	Ile Arg Ser Leu Lys	Lys		
		415		420		425
	Ser Gly Lys Leu	Trp Leu Asp Ala Tyr	Leu His Lys ***	Ser	His	
		430		435		440
30	Ser Pro Arg Asp	Thr Val Gly Lys Gly	Cys Arg Trp Gly Asp	Gly		
		445		450		455
	Gln Arg Asn Asp	Gly Leu Leu Phe ***	Gly Val Ser Glu Arg	Leu		
		460		465		470
	Val Tyr Ala Leu	Leu Thr Asp Pro Met	Leu Asp Leu Ser Pro	Phe		
35		475		480		485
	Leu Leu Ser Phe	Phe Ser Ser His Leu	Pro His Ser Thr Leu	Pro		
		490		495		500
	Ser Trp Asp Leu	Pro Ser Leu Ser Arg	Gln Pro Ser Ala Met	Ala		
		505		510		515
40	Leu Pro Val Pro	Pro Ser Pro Phe Phe	Gln Gly Ala Glu Arg	Trp		
		520		525		530
	Pro Pro Gly Val	Ala Leu Ser Tyr Leu	His Ser Leu Pro Leu	Lys		
		535		540		545
	Met Gly Gly Asp	Gln Arg Ser Met Gly	Leu Ala Cys Glu Ser	Pro		
45		550		555		560
	Leu Ala Ala Trp	Ser Leu Gly Ile Thr	Pro Ala Leu Val Leu	Gln		
		565		570		575
	Met Leu Leu Gly	Phe Ile Gly Ala Gly	Pro Ser Arg Ala Gly	Pro		
		580		585		590
50	Leu Thr Leu Pro	Ala Trp Leu His Ser	Pro *** Arg Leu Pro	Leu		
		595		600		605
	Val His Pro Phe	Ile Glu Arg Pro Ala	Leu Leu Gln Ser Ser	Gly		
		610		615		620
	Leu Pro Pro Ala	Ala Arg Leu Ser Thr	Arg Gly Leu Ser ***	Asp		
55		625		630		635
	Val Gln Gly Pro	Arg Pro Ala Gly Thr	Ala Ser Pro Asn Leu	Gly		
		640		645		650
	Pro Trp Lys Ser	Pro Pro Pro His His	*** Ser Ala Leu Thr	Leu		
		655		660		665
60	Gly Phe His Gly	Pro His Ser Thr Ala	Ser Pro Thr ***	Ala	Cys	
		670		675		680
	Asp Leu Gly Thr	Lys Gly Gly Val Pro	Arg Leu Leu ***	Leu	Ser	

685 - 690 695
 Arg Gly Ser Gly His Val Gln Gly Gly Ala Gly Trp Pro Gly Gly
 700 705 710
 5 Ser Ala His Pro Pro Ala Phe Pro Gln Gly Val Leu Arg Ser Lys
 715 720 725
 Ile Leu Glu Gln Ser Asp Pro Ser Pro Lys Ala Leu Leu Ser Ala
 730 735 740
 Gly Gln Cys Gln Pro Ile Pro Gly His Leu Ala Pro Gly Asp Val
 745 750 755
 10 Gly Pro Xxx

(2) INFORMATION FOR SEQ ID NO:45:

15 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 746 nucleic acids
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: not relevant
 20 (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

25 CGTATGTCAC TCCATTCCAA ACTCGTTCAT GGTATCATAA ATATCAACAC ATTTACGCTC 60
 CACTCCTCTA TGGTATTTAC AACTCAAAT ATCGTACTCA AGATTGGGAA GCTTTTGTA 120
 AGGATGGTAA AAATGGTGCA ATTCGTGTTA GTGTCGCCAC AAATTTCGAT AAGGCCGCTT 180
 ACGTCATTGG TAAATTGTCT TTTGTTTTCT TCCGTTTCAT CCTTCCACTC CGTTATCATA 240
 30 GCTTTACAGA TTTAATTTGT TATTTCTCA TTGCTGAATT CGTCTTGGT TGGTATCTCA 300
 CAATTAATTT CCAAGTTAGT CATGTCGCTG AAGATCTCAA ATTCTTTGCT ACCCTGAAA 360
 GACCAGATGA ACCATCTCAA ATCAATGAAG ATTGGGCAAT CCTTCAACTT AAACTACTC 420
 AAGATTATGG TCATGGTTCA CTCCTTTGTA CTTTTTTAG TGGTCTTTA AATCATCAAG 480
 TTGTTTCATCA TTTATTCCCA TCAATTGCTC AAGATTCTA CCCACAACTT GTACCAATTG 540
 35 TAAAGAAGT TTGTAAAGAA CATAACATTA CTTACCACAT TAAACCAAAC TTCCTGAAG 600
 CTATTATGTC ACACATTAAT TACCTTTACA AAATGGGTAA TGATCCAGAT TATGTTAAA 660
 AACCATTAGC CTCAAAGAT GATTAAATGA AATAACTTAA AAACCAATTA TTTACTTTTG 720
 ACAAACAGTA ATATTAATAA ATACAA 746

40 (2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 227 amino acids
 (B) TYPE: amino acid
 45 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

Tyr Val Thr Pro Phe Gln Thr Arg Ser Trp Tyr His Lys Tyr Gln
 1 5 10 15
 55 His Ile Tyr Ala Pro Leu Leu Tyr Gly Ile Tyr Thr Leu Lys Tyr
 20 25 30
 Arg Thr Gln Asp Trp Glu Ala Phe Val Lys Asp Gly Lys Asn Gly
 35 40 45
 Ala Ile Arg Val Ser Val Ala Thr Asn Phe Asp Lys Ala Ala Tyr
 50 55 60
 60 Val Ile Gly Lys Leu Ser Phe Val Phe Arg Phe Ile Leu Pro
 65 70 75
 Leu Arg Tyr His Ser Phe Thr Asp Leu Ile Cys Tyr Phe Leu Ile
 80 85 90
 65 Ala Glu Phe Val Phe Gly Trp Tyr Leu Thr Ile Asn Phe Gln Val
 95 100 105

Ser His Val Ala Glu Asp Leu Lys Phe Phe Ala Thr Pro Glu Arg
 110 115 120
 Pro Asp Glu Pro Ser Gln Ile Asn Glu Asp Trp Ala Ile Leu Gln
 125 130 135
 5 Leu Lys Thr Thr Gln Asp Tyr Gly His Gly Ser Leu Leu Cys Thr
 140 145 150
 Phe Phe Ser Gly Ser Leu Asn His Gln Val Val His His Leu Phe
 155 160 165
 10 Pro Ser Ile Ala Gln Asp Phe Tyr Pro Gln Leu Val Pro Ile Val
 170 175 180
 Lys Glu Val Cys Lys Glu His Asn Ile Thr Tyr His Ile Lys Pro
 185 190 195
 Asn Phe Thr Glu Ala Ile Met Ser His Ile Asn Tyr Leu Tyr Lys
 200 205 210
 15 Met Gly Asn Asp Pro Asp Tyr Val Lys Lys Pro Leu Ala Ser Lys
 215 220 225
 Asp Asp ***

20 (2) INFORMATION FOR SEQ ID NO 47:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 494 nucleic acids
 (B) TYPE: nucleic acid
 25 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

TTTTGGAAGG NTCCAAGTTN ACCACGGANT NGGCAAGTTN ACGGGGCGGA AANCGGTTTT 60
 CCCCCCAAGC CTTTGTGCGA CTGGTTCTGT GGTGGCTTCC AGTACCAAGT CGACCACCAC 120
 35 TTATTCCCA GCCTGCCCCG ACACAATCTG GCCAAGACAC ACGCACTGGT CGAATCGTTC 180
 TGCAAGGAGT GGGGTGTCCA GTACCACGAA GCCGACCTCG TGGACGGGAC CATGGAAGTC 240
 TTGCACCATT TGGCAGCGT GGCCGGCGAA TTCGTCGTGG ATTTGTACG CGACGGACCC 300
 GCCATGTAAT CGTCGTTCTG GACGATGCAA GGGTTCACGC ACATCTACAC AACTCACTC 360
 ACACAAC TAGTAACTCGT ATAGAATTCG GTGTCGACCT GGACCTGTT TGA CTGGTTG 420
 40 GGGATAGGGT AGGTAGGCGG ACGCGTGGGT CGNCCCCGGG AATTCTGTGA CCGGTACCTG 480
 GCGCGCTNA AAGT 494

45 (2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 87 amino acids
 (B) TYPE: amino acid
 50 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

Phe Trp Lys Xxx Pro Ser Xxx Pro Arg Xxx Xxx Gln Val Xxx Gly
 1 5 10 15
 60 Ala Glu Xxx Gly Phe Pro Pro Lys Pro Phe Val Asp Trp Phe Cys
 20 25 30
 Gly Gly Phe Gln Tyr Gln Val Asp His His Leu Phe Pro Ser Leu
 35 40 45
 Pro Arg His Asn Leu Ala Lys Thr His Ala Leu Val Glu Ser Phe
 50 55 60
 65 Cys Lys Glu Trp Gly Val Gln Tyr His Glu Ala Asp Leu Val Asp
 65 70 75

Gly Thr Met Glu Val Leu His His Leu Gly Ser Val Ala Gly Glu
 65 70 75
 Phe Val Val Asp Phe Val Arg Asp Gly Pro Ala Met
 80 85

5

10

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:

15

- (A) LENGTH: 520 nucleic acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

25

GGATGGAGTT CGTCTGGATC GCTGTGCGCT ACGCGACGTG GTTTAAGCGT CATGGGTGCG 60
 CTTGGGTACA CGCCGGGGCA GTCGTTGGGC ATGTACTTGT GCGCCTTTGG TCTCGGCTGC 120
 ATTTACATTT TTCTGCAGTT CGCCGTAAGT CACACCCATT TGCCCGTGAG CAACCCGGAG 180
 GATCAGCTGC ATTGGCTCGA GTACGCGCGG ACCACACTGT GAACATCAGC ACCAAGTCGT 240
 GGTTCGTCAC ATGGTGGATG TCGAACCTCA ACTTTCAGAT CGAGCACCAC CTTTTCCCCA - 300
 CGGCGCCCCA GTTCCGTTTC AAGGAGATCA GCCGCGCGCT CGAGGCCCTC TTCAAGCGCC 360
 ACGGTCTCCC TTACTACGAC ATGCCCTACA CGAGCGCCGT CTCCACCACC TTTGCCAACC 420
 TCTACTCCGT CGGCCATTCC GTCGGCGACG CCAAGCGCGA CTAGCCTCTT TTCCTAGACC 480
 TTAATTCCCC ACCCCACCCC ATGTTCTGTC TTCCTCCGCG 520

35

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:

40

- (A) LENGTH: 153 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

50

55

60

65

Met Glu Phe Val Trp Ile Ala Val Arg Tyr Ala Thr Trp Phe Lys
 1 5 10 15
 Arg His Gly Cys Ala Trp Val His Ala Gly Ala Val Val Gly His
 20 25 30
 Val Leu Val Arg Leu Trp Ser Arg Leu His Leu His Phe Ser Ala
 35 40 45
 Val Arg Arg Lys Ser His Pro Phe Ala Arg Glu Gln Pro Gly Gly
 50 55 60
 Ser Ala Ala Leu Ala Arg Val Arg Ala Asp His Thr Val Asn Ile
 65 70 75
 Ser Thr Lys Ser Trp Phe Val Thr Trp Trp Met Ser Asn Leu Asn
 80 85 90
 Phe Gln Ile Glu His His Leu Phe Pro Thr Ala Pro Gln Phe Arg
 95 100 105
 Phe Lys Glu Ile Ser Pro Arg Val Glu Ala Leu Phe Lys Arg His
 110 115 120
 Gly Leu Pro Tyr Tyr Asp Met Pro Tyr Thr Ser Ala Val Ser Thr
 125 130 135
 Thr Phe Ala Asn Leu Tyr Ser Val Gly His Ser Val Gly Asp Ala

Lys Arg Asp 140 145 150

5

(2) INFORMATION FOR SEQ ID NO:51:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 429 nucleic acids
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: nucleic acid

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

ACGCGTCCGC CCACGCGTCC GCCGCGAGCA ACTCATCAAG GAAGGCTACT TTGACCCCTC 60
 GCTCCCGCAC ATGACGTACC GCGTGGTCGA GATTGTTGTT CTCTTCGTGC TTTCTTTTG 120
 GCTGATGGGT CAGTCTTCAC CCCTCGCGCT CGCTCTCGGC ATTGTCGTCA GCGGCATCTC 180
 TCAGGGTCCG TCGGCTGGG TAATGCATGA GATGGGCCAT GGGTCGTTCA CTGGTGTTCAT 240
 TTGGCTTGAC GACCGGTTGT GCGAGTTCTT TTACGGCGTT GGTGTGGCA TGAGCGGTCA 300
 TTA CTGAAA AACCAGCACA GCAAACACCA CGCAGCGCCA AACCGGCTCG AGCAGATGT 360
 AGATCTCAAC ACCTTGCCAT TGGTGGCCTT CAACGAGCGC GTCGTGCGCA AGGTCCGACC 420

(2) INFORMATION FOR SEQ ID NO:52:

30

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 125 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: linear

35

- (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

40

Arg Val Arg Pro Arg Val Arg Arg Glu Gln Leu Ile Lys Glu Gly
 1 5 10 15
 Tyr Phe Asp Pro Ser Leu Pro His Met Thr Tyr Arg Val Val Glu
 20 25 30
 Ile Val Val Leu Phe Val Leu Ser Phe Trp Leu Met Gly Gln Ser
 35 40 45
 Ser Pro Leu Ala Leu Ala Leu Gly Ile Val Val Ser Gly Ile Ser
 50 55 60
 Gln Gly Arg Cys Gly Trp Val Met His Glu Met Gly His Gly Ser
 65 70 75
 Phe Thr Gly Val Ile Trp Leu Asp Asp Arg Leu Cys Glu Phe Phe
 80 85 90
 Tyr Gly Val Gly Cys Gly Met Ser Gly His Tyr Trp Lys Asn Gln
 95 100 105
 His Ser Lys His His Ala Ala Pro Asn Arg Leu Glu His Asp Val
 110 115 120
 Asp Leu Asn Thr Leu Pro Leu Val Ala Phe Asn Glu Arg Val Val
 125
 Arg Lys Val Arg Pro

What is claimed is:

1. A nucleic acid construct comprising:

One or more nucleotide sequences depicted in a SEQ ID NO: selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3 and SEQ ID NO:5,
5 wherein said one or more nucleotide sequences is linked to a heterologous nucleotide sequence.

2. A nucleic acid construct comprising:

One or more nucleotide sequences depicted in a SEQ ID NO: selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3 and SEQ ID NO:5,
10 wherein said one or more nucleotide sequences is operably associated with an expression control sequence functional in a plant cell.

3. The nucleic acid construct according to claim 2, wherein said nucleotide
15 sequence has an average A + T content of less than about 60%.

4. The nucleic acid construct according to claim 2, wherein said nucleotide sequence is derived from a fungus.

20 5. The nucleic acid construct according to claim 4, wherein said fungus is of the genus *Mortierella*.

6. The nucleic acid construct according to claim 5, wherein said fungus is of the species *alpina*.

25 7. A nucleic acid construct comprising:

A nucleotide sequence which encodes a polypeptide comprising an amino acid sequence depicted in SEQ ID NO:2, wherein said nucleotide sequence is

operably associated with a transcription or an expression control sequence function in a plant cell, wherein said nucleotide sequence encodes a functionally active polypeptide which desaturates a fatty acid molecule at carbon 6 from the carboxyl end of said fatty acid molecule.

5

8. A nucleic acid construct comprising:

A nucleotide sequence which encodes a polypeptide comprising an amino acid sequence depicted in SEQ ID NO:4, wherein said nucleotide sequence is operably associated with a transcription or an expression control sequence functional in a plant cell, wherein said nucleotide sequence encodes a functionally active polypeptide which desaturates a fatty acid molecule at carbon 12 from the carboxyl end of said fatty acid molecule.

10

9. A nucleic acid construct comprising:

A nucleotide sequence which encodes a polypeptide comprising an amino acid sequence depicted in SEQ ID NO:6, wherein said nucleotide sequence is operably associated with a transcription or an expression control sequence function in a plant cell, wherein said nucleotide sequence encodes a functionally active polypeptide which desaturates a fatty acid molecule at carbon 5 from the carboxyl end of said fatty acid molecule.

15

20

10. A nucleic acid construct comprising:

at least one nucleotide sequence which encodes a functionally active desaturase having an amino acid sequence depicted in a SEQ ID NO: selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:6, wherein said nucleotide sequence is operably associated with a promoter functional in a plant cell.

25

11. The nucleic acid construct according to claim 10, wherein said plant cell is a seed cell.

5 12. The nucleic acid construct according to claim 11, wherein said seed cell is an embryo cell.

13. A recombinant plant cell comprising:

10 At least one copy of a DNA sequence which encodes at least one functionally active *Mortierella alpina* fatty acid desaturase which results in the production of a polyunsaturated fatty acid, wherein said fatty acid desaturase has an amino acid sequence as depicted in a SEQ ID NO: selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, and SEQ ID NO:6, wherein said cell was transformed with a vector comprising said DNA sequence, and wherein said DNA sequence is operably associated with an expression control
15 sequence.

14. The recombinant plant cell of claim 13, wherein said polyunsaturated fatty acid is selected from the group consisting of LA, ARA, GLA, DGLA, SDA and EPA.

20

15. The recombinant plant cell of claim 13, wherein said recombinant plant cell is enriched in a fatty acid selected from the group consisting of 18:1, 18:2, 18:3 and 18:4.

25 16. The recombinant plant cell of claim 15, wherein said plant cell is selected from the group consisting of *Brassica*, soybean, safflower, corn, flax, and sunflower.

17. The recombinant plant cell according to claim 16, wherein said expression control sequence is endogenous to said plant cell.

18. One or more plant oils expressed by said recombinant plant cell of claim 16.

5

19. A method for obtaining altered long chain polyunsaturated fatty acid biosynthesis comprising the steps of:

growing a plant having cells which contain a transgene encoding a transgene expression product which desaturates a fatty acid molecule at carbon
10 5 from the carboxyl end of said fatty acid molecule, wherein said transgene is operably associated with an expression control sequence, under conditions whereby said transgene is expressed, whereby long chain polyunsaturated fatty acid biosynthesis in said cells is altered.

15 20. A method for obtaining altered long chain polyunsaturated fatty acid biosynthesis comprising the steps of:

growing a plant having cells which contain one or more transgenes, derived from a fungus or algae, which encodes a transgene expression product which desaturates a fatty acid molecule at a carbon selected from the group
20 consisting of carbon 5, carbon 6 and carbon 12 from the carboxyl end of said fatty acid molecule, wherein said one or more transgenes is operably associated with an expression control sequence, under conditions whereby said one or more transgenes is expressed, whereby long chain polyunsaturated fatty acid biosynthesis in said cells is altered.

25

21. The method according to claims 19 or 20, wherein said long chain polyunsaturated fatty acid is selected from the group consisting of LA, ARA, GLA, DGLA, SDA and EPA.

22. A plant oil or fraction thereof produced according to the method of claims
19 or 20.
- 5 23. A method of treating or preventing malnutrition comprising administering
said plant oil of claim 22 to a patient in need of said treatment or prevention
in an amount sufficient to effect said treatment or prevention.
24. A pharmaceutical composition comprising said plant oil or fraction of claim
22 and a pharmaceutically acceptable carrier.
- 10
25. The pharmaceutical composition of claim 24, wherein said pharmaceutical
composition is in the form of a solid or a liquid.
26. The pharmaceutical composition of claim 25, wherein said pharmaceutical
15 composition is in a capsule or tablet form.
27. The pharmaceutical composition of claim 24 further comprising at least one
nutrient selected from the group consisting of a vitamin, a mineral, a
carbohydrate, a sugar, an amino acid, a free fatty acid, a phospholipid, an
20 antioxidant, and a phenolic compound.
28. A nutritional formula comprising said plant oil or fraction thereof of claim
22.
- 25 29. The nutritional formula of claim 28, wherein said nutritional formula is
selected from the group consisting of an infant formula, a dietary
supplement, and a dietary substitute.

30. The nutritional formula of claim 29, wherein said infant formula, dietary supplement or dietary supplement is in the form of a liquid or a solid.

31. An infant formula comprising said plant oil or fraction thereof of claim 22.

5

32. The infant formula of claim 31 further comprising at least one macronutrient selected from the group consisting of coconut oil, soy oil, canola oil, mono- and diglycerides, glucose, edible lactose, electro dialysed whey, electro dialysed skim milk, milk whey, soy protein, and other protein hydrolysates.

10

33. The infant formula of claim 32 further comprising at least one vitamin selected from the group consisting of Vitamins A, C, D, E, and B complex; and at least one mineral selected from the group consisting of calcium, magnesium, zinc, manganese, sodium, potassium, phosphorus, copper, chloride, iodine, selenium, and iron.

15

34. A dietary supplement comprising said plant oil or fraction thereof of claim 22.

20

35. The dietary supplement of claim 34 further comprising at least one macronutrient selected from the group consisting of coconut oil, soy oil, canola oil, mono- and diglycerides, glucose, edible lactose, electro dialysed whey, electro dialysed skim milk, milk whey, soy protein, and other protein hydrolysates.

25

36. The dietary supplement of claim 35 further comprising at least one vitamin selected from the group consisting of Vitamins A, C, D, E, and B complex; and at least one mineral selected from the group consisting of calcium,

magnesium, zinc, manganese, sodium, potassium, phosphorus, copper, chloride, iodine, selenium, and iron.

5 37. The dietary supplement of claim 34 or claim 36, wherein said dietary supplement is administered to a human or an animal.

38. A dietary substitute comprising said plant oil or fraction thereof of claim 22.

10 39. The dietary substitute of claim 38 further comprising at least one macronutrient selected from the group consisting of coconut oil, soy oil, canola oil, mono- and diglycerides, glucose, edible lactose, electro dialysed whey, electro dialysed skim milk, milk whey, soy protein, and other protein hydrolysates.

15 40. The dietary substitute of claim 39 further comprising at least one vitamin selected from the group consisting of Vitamins A, C, D, E, and B complex; and at least one mineral selected from the group consisting of calcium, magnesium, zinc, manganese, sodium, potassium, phosphorus, copper, chloride, iodine, selenium, and iron.

20

41. The dietary substitute of claim 38 or claim 40, wherein said dietary substitute is administered to a human or animal.

25 42. A method of treating a patient having a condition caused by insufficient intake or production of polyunsaturated fatty acids comprising administering to said patient said dietary substitute of claim 38 or said dietary supplement of claim 34 in an amount sufficient to effect said treatment.

43. The method of claim 42, wherein said dietary substitute or said dietary supplement is administered enterally or parenterally.

44. A cosmetic comprising said plant oil or fraction thereof of claim 22.

5

45. The cosmetic of claim 44, wherein said cosmetic is applied topically.

46. The pharmaceutical composition of claim 24, wherein said pharmaceutical composition is administered to a human or an animal.

10

47. An animal feed comprising said plant oil or fraction thereof of claim 22.

48. An isolated nucleotide sequence comprising the nucleotide sequence selected from the group consisting of SEQ ID NO:38 - SEQ ID NO:44 wherein said nucleotide sequence is expressed in a plant cell.

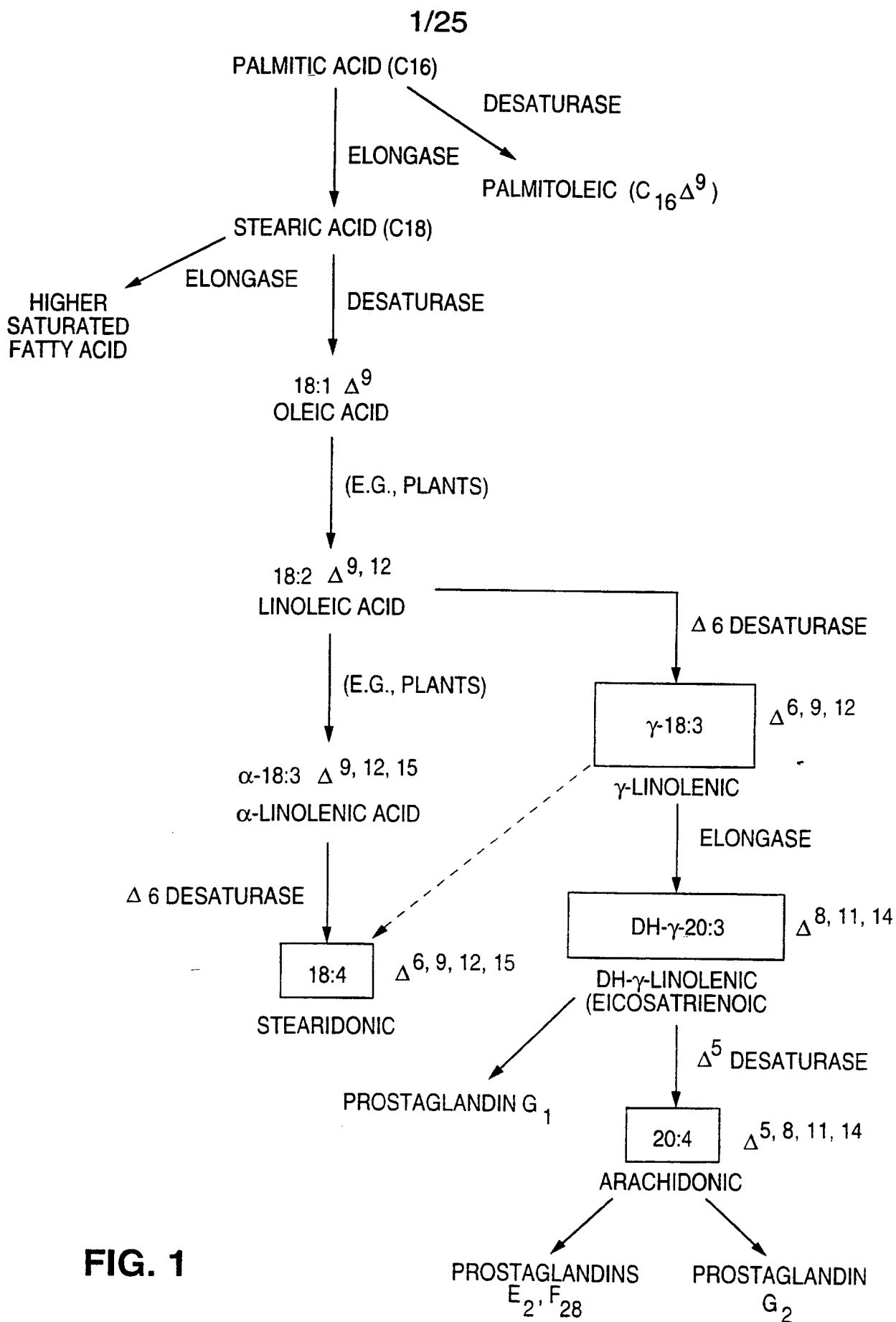
15

49. The method of claim 20 wherein said fungus is *Mortierella species*.

50. The method of claim 49 wherein said fungus is *Mortierella alpina*.

20

51. An isolated nucleotide sequence selected from the group consisting of SEQ ID NO:49 - SEQ ID NO:50 wherein said sequence is expressed in a plant cell.



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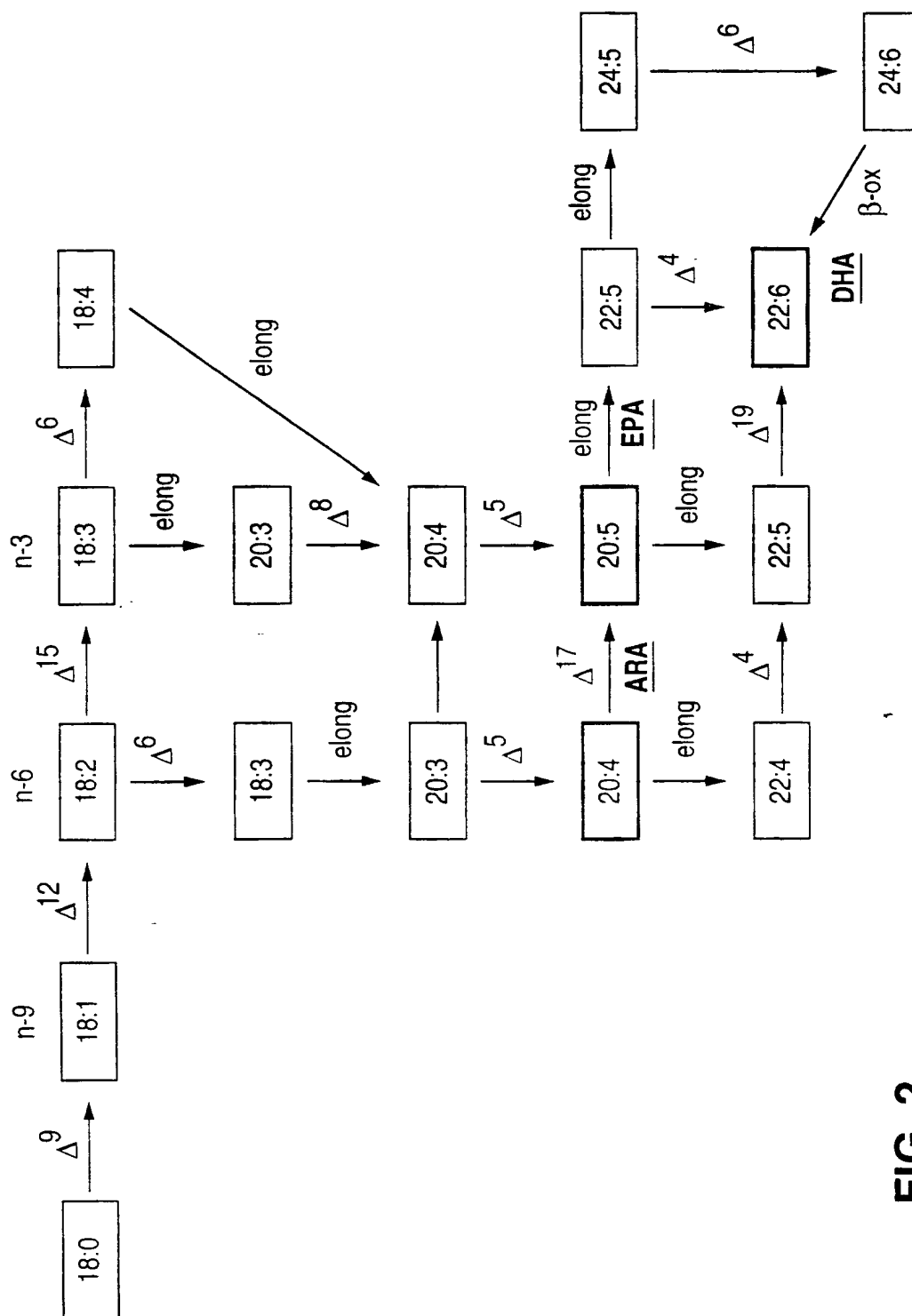


FIG. 2

60
 *
 CGACACTCCT TCCTTCTTCT CACCCGTCCT AGTCCCCTTC AACCCCCCTC TTTGACAAAG
 ACAACAAACC ATG GCT GCT GCT CCC AGT GTG AGG ACG TTT ACT CGG GCC GAG
 Met Ala Ala Pro Ser Val Arg Thr Phe Thr Arg Ala Glu
 120
 *
 GTT TTG AAT GCC GAG GCT CTG AAT GAG GGC AAG GAT GCC GAG GCA
 Val Leu Asn Ala Glu Ala Leu Asn Glu Gly Lys Asp Ala Glu Ala
 180
 *
 CCC TTC TTG ATG ATC ATC GAC AAC AAG GTG TAC GAT GTC CGC GAG TTC
 Pro Phe Leu Met Ile Ile Asp Asn Lys Val Tyr Asp Val Arg Glu Phe
 240
 *
 GTC CCT GAT CAT CCC GGT GGA AGT GTG ATT CTC ACG CAC GTT GGC AAG
 Val Pro Asp His Pro Gly Gly Ser Val Ile Leu Thr His Val Gly Lys
 300
 *
 GAC GGC ACT GAC GTC TTT GAC ACT TTT CAC CCC GAG GCT GCT TGG GAG
 Asp Gly Thr Asp Val Phe Asp Thr Phe His Pro Glu Ala Ala Trp Glu
 360
 *
 ACT CTT GCC AAC TTT TAC GTT GGT GAT ATT GAC GAG AGC GAC CGC GAT
 Thr Leu Ala Asn Phe Tyr Val Gly Asp Ile Asp Glu Ser Asp Arg Asp
 ATC AAG AAT GAT GAC TTT GCG GCC GAG GTC CGC AAG CTG CGT ACC TTG
 Ile Lys Asn Asp Asp Phe Ala Ala Glu Val Arg Lys Leu Arg Thr Leu

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FIG. 3A

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TTC	CAG	TCT	CTT	GGT	TAC	TAC	GAT	TCT	TCC	AAG	GCA	TAC	TAC	GCC	TTC
Phe	Gln	Ser	Leu	Gly	Tyr	Tyr	Asp	Ser	Ser	Lys	Ala	Tyr	Tyr	Ala	Phe
420 *															
AAG	GTC	TCG	TTC	AAC	CTC	TGC	ATC	TGG	GGT	TTG	TCG	ACG	GTC	ATT	GTG
Lys	Val	Ser	Phe	Asn	Leu	Cys	Ile	Trp	Gly	Leu	Ser	Thr	Val	Ile	Val
480 *															
GCC	AAG	TGG	GGC	CAG	ACC	TCG	ACC	CTC	GCC	AAC	GTG	CTC	TCG	GCT	GCG
Ala	Lys	Trp	Gly	Gln	Thr	Ser	Thr	Leu	Ala	Asn	Val	Leu	Ser	Ala	Ala
540 *															
CTT	TTG	GGT	CTG	TTC	TGG	CAG	CAG	TGC	GGA	TGG	TTG	GCT	CAC	GAC	TTT
Leu	Leu	Gly	Leu	Phe	Trp	Gln	Gln	Cys	Gly	Trp	Leu	Ala	His	Asp	Phe
600 *															
TTG	CAT	CAC	CAG	GTC	TTC	CAG	GAC	CGT	TTC	TGG	GGT	GAT	CTT	TTC	GGC
Leu	His	His	Gln	Val	Phe	Gln	Asp	Arg	Phe	Trp	Gly	Asp	Leu	Phe	Gly
660 *															
GCC	TTC	TTG	GGA	GGT	GTC	TGC	CAG	GGC	TTC	TCG	TCC	TCG	TGG	TGG	AAG
Ala	Phe	Leu	Gly	Gly	Val	Cys	Gln	Gly	Phe	Ser	Ser	Ser	Trp	Trp	Lys
720 *															
GAC	AAG	CAC	AAC	ACT	CAC	CAC	GCC	GCC	CCC	AAC	GTC	CAC	GGC	GAG	GAT
Asp	Lys	His	Asn	Thr	His	His	Ala	Ala	Pro	Asn	Val	His	Gly	Glu	Asp
780 *															

FIG. 3B

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CCC	GAC	ATT	GAC	ACC	CAC	CCT	CTG	TTG	ACC	TGG	AGT	GAG	CAT	GCG	TTG
Pro	Asp	Ile	Asp	Thr	His	Pro	Leu	Leu	Thr	Trp	Ser	Glu	His	Ala	Leu
GAG	ATG	TTC	TCG	GAT	GTC	CCA	GAT	GAG	GAG	CTG	ACC	CGC	ATG	TGG	TCG
Glu	Met	Phe	Ser	Asp	Val	Pro	Asp	Glu	Glu	Leu	Thr	Arg	Met	Trp	Ser
840 *															
CGT	TTC	ATG	GTC	CTG	AAC	CAG	ACC	TGG	TTT	TAC	TTC	CCC	ATT	CTC	TCG
Arg	Phe	Met	Val	Leu	Asn	Gln	Thr	Trp	Phe	Tyr	Phe	Pro	Ile	Leu	Ser
900 *															
TTT	GCC	CGT	CTC	TCC	TGG	TGC	CTC	CAG	TCC	ATT	CTC	TTT	GTG	CTG	CCT
Phe	Ala	Arg	Leu	Ser	Trp	Cys	Leu	Gln	Ser	Ile	Leu	Phe	Val	Leu	Pro
960 *															
AAC	GGT	CAG	GCC	CAC	AAG	CCC	TCG	GGC	GCG	CGT	GTG	CCC	ATC	TCG	TTG
Asn	Gly	Gln	Ala	His	Lys	Pro	Ser	Gly	Ala	Arg	Val	Pro	Ile	Ser	Leu
1020 *															
GTC	GAG	CAG	CTG	TCG	CTT	GCG	ATG	CAC	TGG	ACC	TGG	TAC	CTC	GCC	ACC
Val	Glu	Gln	Leu	Ser	Leu	Ala	Met	His	Trp	Thr	Trp	Tyr	Leu	Ala	Thr
ATG	TTC	CTG	TTC	ATC	AAG	GAT	CCC	GTC	AAC	ATG	CTG	GTG	TAC	TTT	TTG
Met	Phe	Leu	Phe	Ile	Lys	Asp	Pro	Val	Asn	Met	Leu	Val	Tyr	Phe	Leu
1080 *															
GTG	TCG	CAG	GCG	GTG	TGC	GGA	AAC	TTG	TTG	GCG	ATC	GTG	TTC	TCG	CTC
Val	Ser	Gln	Ala	Val	Cys	Gly	Asn	Leu	Leu	Ala	Ile	Val	Phe	Ser	Leu

FIG. 3C

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1140
 AAC CAC AAC GGT ATG CCT GTG ATC TCG AAG GAG GCG GTC GAT ATG
 Asn His Asn Gly Met Pro Val Ile Ser Lys Glu Glu Ala Val Asp Met
 1200
 GAT TTC TTC ACG AAG CAG ATC ATC ACG GGT CGT GAT GTC CAC CCG GGT
 Asp Phe Phe Thr Lys Gln Ile Ile Ile Thr Gly Arg Asp Val His Pro Gly
 1260
 CTA TTT GCC AAC TGG TTC ACG GGT GGA TTG AAC TAT CAG ATC GAG CAC
 Leu Phe Ala Asn Trp Phe Thr Gly Gly Gly Leu Asn Tyr Gln Ile Glu His
 1320
 CAC TTG TTC CCT TCG ATG CCT CGC CAC AAC TTT TCA AAG ATC CAG CCT
 His Leu Phe Pro Ser Met Pro Arg His Asn Phe Ser Lys Ile Gln Pro
 1380
 GCT GTC GAG ACC CTG TGC AAA AAG TAC AAT GTC CGA TAC CAC ACC ACC
 Ala Val Glu Thr Leu Cys Lys Lys Tyr Tyr Val Val Arg Tyr His Thr Thr
 1440
 GGT ATG ATC GAG GGA ACT GCA GAG GTC TTT AGC CGT CTG AAC GAG GTC
 Gly Met Ile Glu Gly Thr Ala Glu Val Phe Ser Arg Leu Asn Glu Val
 TCC AAG GCT GCC TCC AAG ATG GGT AAG GCG CAG TAA AAAAAA AAACAAGGAC
 Ser Lys Ala Ala Ser Lys Met Gly Lys Ala Gln

FIG. 3D

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1500
GTTTTTTTC GCCAGTGCCT GTGCCCTGTGC CTGCTTCCCT^{*} TGTC AAGTCG AGCGTTTCTG
1560
GAAAGGATCG TTCAGTGCAG TATCATCATT CTCCTTTTAC^{*} CCCCCGCTCA TATCTCATTC
ATTTCCTTA TTAACAACCT TGTTCCCCCCTTCACCG

FIG. 3E

Ma524 EVRKLRTL FQSLGYDSSKAYYAFKVSFNLCIWGLSTVIIVAKWGQTSSTLANVLSAALLGL 90
 ATTS4723 - - - - - VTLY-TLAFVAMSLGVLYGVLACPSVXPHQIAAGLLGL 38
 12-5 - - - - - GVLVGLACTSVFAHQIAAALLGL 24
 T42806 - - - - - GXX - - - - - 4
 W28140 - - - - - - - - - - - 1
 R05219 - - - - - C - - - - - 2
 W53753 - - - - - - - - - - - 1

Ma524 FWQQCGWLAHDFLHHQVFQDRFWGDL-FGAF LGGVC-QGFSSSWWKDKHNTTHH AAPNVHGE 119
 ATTS4723 LWIQSAYIGXD SGHYVIMSNKSNX-FAQL LSGNCLTG I IAWWKWTHNHH LACN SLDY 97
 12-5 LWIQSAYIGHD SGHYVIMSNKSNR-FAQL LSGNCLTG I IAWWKWTHNHH LACN SLDY 83
 T42806 - - - - - - - - - - - 4
 W28140 - - - - - - - - - - - 1
 R05219 - - - - - - - - - - - 2
 W53753 - - - - - - - - - - - 1

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Ma524 DPDI DTHPLLTWSEHALEMFS DVPDEELTRMWS- - - - - RFMV LNQTWFYFPILSFARLSW 174
 ATTS4723 GPNLQH IIP - - - - - 105
 12-5 DPDLQH IIPVFAVSTK- - - - - FSSLT SRFYDRKLTFGPVARFLVSYQHFTYYPVMC FGRINL 140
 T42806 - - - - - - - - - - - 4
 W28140 - - - - - - - - - - - 1
 R05219 - - - - - - - - - - - 2
 W53753 - - - - - - - - - - - 1

FIG. 4A

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Ma524	CLQSILFVLPNGQAHKPSGARVPI SLVEQLSLAM	-----HWTWYLATMFLFIKDPVNMLV	229
ATTS4723		W W	105
12-5	FIQTFLLFISKRE	-----VPRALNFAGILV-----FWTWIF--PLLVSCLPNWPERF	185
T42806		-----NFAGILV-----FFTVIF--PLLVSCLPNWPERF	29
W28140		-----PATEVGGLAWMIT-Y-RFFLTYVPLLGLKAF	33
R05219		-----RHEAARGGTRLAYMLVCMQWTDL--LWAAS Y RFFLSYSPFYGATGT	2
W53753			48
Ma524	YFLVSQAVCGNLLAIVFSLNHNGMPVISKEEAVDMDFFTKQIITGRDVPHPGLFANWFTGG		289
ATTS4723			105
12-5	FFVFTSFTVTA	LQHIIQFIQF	244
T42806		-----GPPPTGSDWFEKQIAAGTIDISCRSYMDWFFFGG	88
W28140		-----GPPPTGSDWFEKQIAAGTIDISCRSYMDWFFFGG	90
R05219		-----IPMHIDHDRNMDWVSTQLQATCNVHKSAFNDWFSGH	23
W53753		-----SPKSSPTRNMTTSPFIDWLWGG	105
Ma524	LFAVRVLESHWFVWITQMNH	-----IPKEIGHEKHRRDWASSQLAATCNVESP	349
ATTS4723			105
12-5	LNYQIEHHLFPSMPRHNFSKIQPAVETLCKKYNVRYHTTGMIEGTAEVESRLNEVSKAAS		252
T42806			125
W28140			131
R05219			83
W53753			143
Ma524	LQFQLEHH		355
ATTS4723			105
12-5	LQFQLEHHLFPR	LPRLPRCHLRKVS	252
T42806		-----VVGQRGFQRKXNLSX	125
W28140		-----PLVQSLCAKHGIEYQSKPL	131
R05219		-----PLVQSLCAKHGIEYQSKPL	83
W53753		-----PLVQSLCAKHGIEYQSKPL	143
Ma524	KMGKAQ		355
ATTS4723			105
12-5			252
T42806			125
W28140			131
R05219			87
W53753			148

- - A K A A

FIG. 4B

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60
 G T C C C C T G T C G C T G T C G G C A C A C C C C A T C C T C C C T G G C T C C C T C T G C G T T T G T C C T T G G C
 120
 C C A C C G T C T C T C C T C C A C C C T C C G A G A C G A C T G T A A T C A G G A A C C G A C A A T A C
 180
 A C G A T T T C T T T T A C T C A G C A C C A A C T C A A A A T C C T C A A C C G C A A C C C T T T T C A G G A T G
 Met
 G C A C C T C C C A A C A C T A T C G A T G C C G G T T T G A C C C A G C G T C A T A T C A G C
 A l a P r o P r o A s n T h r I l e A s p A l a G l y L e u T h r A r g G l n A r g H i s I l e S e r
 240
 A C C T C G G C C C A A A C T C G G C C A A G C C T G C C T T T C G A G C G C A A C T A C C A G
 T h r S e r A l a P r o A s n S e r A l a L y s P r o A l a P h e G l u A r g A s n T y r G l n
 300
 C T C C C C G A G T T C A C C A T C A A G G A G A T C C G A G A G T G C A T C C C T G C C C A C
 L e u P r o G l u P h e T h r I l e L y s G l u I l e A r g G l u C y s I l e P r o A l a H i s
 360
 T G C T T T G A G C G C T C C G G T C T C C G T C G T G G T C T C T G C C A C G A T T G C C A T C G A T
 C y s P h e G l u A r g S e r G l y L e u A r g G l y L e u C y s H i s V a l A l a I l e A s p
 420
 C T G A C T T G G G C G T C G C T T T C T T G C T G G T G C C A C C A G A T C G A C A A G
 L e u T h r T r p A l a S e r L e u L e u P h e L e u A l a A l a T h r G l n I l e A s p L y s
 T T T G A G A A T C C C T T G A T C C G C T A T T T G G C C T G G C C T G T T A C T G G A T C
 P h e G l u A s n P r o L e u I l e A r g T y r L e u A l a T r p P r o V a l T y r T r p I l e

FIG. 5A

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ATG Met	CAG Gln	GGT Gly	ATT Ile	GTC Val	TGC Cys	ACC Thr	GGT Gly	GTC Val	TGG Trp	GTG Val	CTG Leu	GCT Ala	CAC His	GAG Glu	TGT Cys
			480 *				540 *								
GGT Gly	CAT His	CAG Gln	TCC Ser	TTC Phe	TCG Ser	ACC Thr	TCC Ser	AAG Lys	ACC Thr	CTC Leu	AAC Asn	AAC Asn	ACA Thr	GTT Val	GGT Gly
											600 *				
TGG Trp	ATC Ile	TTG Leu	CAC His	TCG Ser	ATG Met	CTC Leu	TTG Leu	GTC Val	CCC Pro	TAC Tyr	CAC His	TCC Ser	TGG Trp	AGA Arg	ATC Ile
															660 *
TCG Ser	CAC His	TCG Ser	Lys	CAC His	CAC His	AAG Lys	GCC Ala	ACT Thr	GGC Gly	CAT His	ATG Met	ACC Thr	AAG Lys	GAC Asp	CAG Gln
GTC Val	TTT Phe	GTG Val	CCC Pro	AAG Lys	ACC Thr	CGC Arg	TCC Ser	CAG Gln	GTT Val	GGC Gly	TTG Leu	CCT Pro	CCC Pro	AAG Lys	GAG Glu
			720 *												
AAC Asn	GCT Ala	GCT Ala	GCT Ala	GCC Ala	GTT Val	CAG Gln	GAG Glu	GAG Glu	GAC Asp	ATG Met	TCC Ser	GTG Val	CAC His	CTG Leu	GAT Asp
							780 *								
GAG Glu	GAG Glu	GCT Ala	CCC Pro	ATT Ile	GTG Val	ACT Thr	TTG Leu	TTC Phe	TGG Trp	ATG Met	GTG Val	ATC Ile	CAG Gln	TTC Phe	TTG Leu
												840 *			
TTC Phe	GGA Gly	TGG Trp	CCC Pro	GCG Ala	TAC Tyr	CTG Leu	ATT Ile	ATG Met	AAC Asn	GCC Ala	TCT Ser	GGC Gly	CAA Gln	GAC Asp	TAC Tyr

FIG. 5B

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GGC Gly	CGC Arg	TGG Trp	ACC Thr	TCG Ser	CAC His	TTC Phe	CAC his	ACG Thr	TAC Tyr	TCG Ser	CCC Pro	ATC Ile	TTT Phe	GAG Glu	CCC Pro	900*
CGC Arg	AAC Asn	TTT Phe	TTC Phe	GAC Asp	ATT Ile	ATT Ile	ATC Ile	TCG Ser	GAC Asp	CTC Leu	GGT Gly	GTG Val	TTG Leu	GCT Ala	GCC Ala	
CTC Leu	GGT Gly	GCC Ala	CTG Leu	ATC Ile	TAT Tyr	GCC Ala	TCC Ser	ATG Met	CAG Gln	TTG Leu	TCG Ser	CTC Leu	TTG Leu	ACC Thr	GTC Val	
ACC Thr	AAG Lys	TAC Tyr	TAT Tyr	ATT Ile	GTC Val	CCC Pro	TAC Tyr	CTC Leu	TTT Phe	GTC Val	AAC Asn	TTT Phe	TGG Trp	TTG Lru	GTC Val	
CTG Leu	ATC Ile	ACC Thr	TTC Phe	TTG Leu	CAG Gln	CAC His	ACC Thr	GAT Asp	CCC Pro	AAG Lys	CTG Leu	CCC Pro	CAT His	TAC Tyr	CGC Arg	
GAG Glu	GGT Gly	GCC Ala	TGG Trp	AAT Asn	TTC Phe	CAG Gln	CGT Arg	GGA Gly	GCT Ala	CTT Leu	TGC Cys	ACC Thr	GTT Val	GAC Asp	CGC Arg	1140*
TCG Ser	TTT Phe	GGC Gly	AAG Lys	TTC Phe	TTG Leu	GAC Asp	CAT His	ATG Met	TTC Phe	CAC His	GGC Gly	ATT Ile	GTC Val	CAC His	ACC Thr	
CAT His	GTG Val	GCC Ala	CAT His	CAC His	TTG Leu	TTC Phe	TCG Ser	CAA Gln	ATG Met	CCG Pro	TTC Phe	TAC Tyr	CAT His	GCT Ala	GAG Glu	

FIG. 5C

1260 *
 GAA GCT ACC TAT CAT CTC AAG AAA CTG CTG GGA GAG TAC TAT GTG TAC
 Glu Ala Thr Tyr His Leu Lys Lys Leu Leu Gly Glu Tyr Tyr Val Tyr

 1320 *
 GAC CCA TCC CCG ATC GTC GTC GCG GGT GCG AGG TCG TTC CGT GAG TGC
 Asp Pro Ser Pro Ile Val Val Ala Val Trp Arg Ser Phe Arg Glu Cys

 1380 *
 CGA TTC GTG GAG GAT CAG GGA GAC GTG GTC TTT TTC AAG AAG TAA AAA
 Arg Phe Val Glu Asp Gln Gly Asp Val Val Phe Phe Lys Lys

 1440 *
 AAAAGACAAT GGACCACACA CAACCTTGTC TCTACAGACC TACGTATCAT GTAGCCATAC
 CACTTCATAA AAGAACATGA GCTCTAGAGG CGTGTCATTC GCGCCTCC

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FIG. 5D

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10	20	30	40	50	60						
LHHTYTN	IAG	ADPDVSTSEP	DVRR	IKPNQK	WFFVNHINQHM	FVPFLYGLLA	FKVRIQDINI	*			
70	80	90	100	110	120						
LYFVKTNDA	I	RVNPISTWHT	VMFWGGK	AFF	VWYRL	I	VPLQ	YLPLGKVL	LL	FTVADMVSSY	*
130	140	150	160	170	180						
WLALTFQANY	VVEEVQWPLP	DENG	I	IQKDW	AAMQVET	TTQD	YAHDSHLWTS	I	TGSLNYQXV	*	

HHLFPH

FIG. 6

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GCTTCCTCCA GTTCATCCTC CATTTCGCCA CCTGCAATTCT TTACGACCGT TAAGCAAG
 60
 ATG GGA ACG GAC CAA GGA AAA ACC TTC ACC TGG GAA GAG CTG GCG GCC
 met Gly Thr Asp Gln Gly Lys Thr Phe Thr Trp Glu Glu Leu Ala Ala
 120
 CAT AAC ACC AAG GAC GAC CTA CTC TTG GCC ATC CGC GGC AGG GTG TAC
 His Asn Thr Lys Asp Asp Leu Leu Leu Ala Ile Arg Gly Arg Val Tyr
 180
 GAT GTC ACA AAG TTC TTG AGC CGC CAT CAT CCT GGT GGA GTG GAC ACT CTC
 Asp Val Thr Lys Phe Phe Leu Ser Arg Arg His Pro Gly Gly Val Asp Thr Leu
 240
 CTG CTC GGA GCT GGC CGA GAT GTT ACT CCG GTC TTT GAG ATG TAT CAC
 Leu Leu Gly Ala Gly Arg Asp Val Val Thr Pro Val Phe Glu Met Tyr His
 300
 GCG TTT GGG GCT GCA GAT GCC ATT ATG AAG AAG TAC TAT GTC GGT ACA
 Ala Phe Gly Ala Ala Asp Ala Ile Met Lys Lys Tyr Tyr Val Gly Thr
 360
 CTG GTC TCG AAT GAG CTG CCC ATC TTC CCG GAG CCA ACG GTG TTC CAC
 Leu Val Ser Asn Glu Glu Pro Ile Phe Pro Glu Glu Thr Val Phe His
 AAA ACC ATC AAG ACG AGA GTC GAG GGC TAC TTT ACG GAT CGG AAC ATT
 Lys Thr Ile Lys Thr Arg Val Glu Gly Tyr Phe Thr Asp Arg Asn Ile

FIG. 7A

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GAT	CCC	AAG	AAT	AGA	CCA	GAG	ATC	TGG	GGA	CGA	TAC	GCT	CTT	ATC	TTT
Asp	Pro	Lys	Asn	Arg	Pro	Glu	Ile	Trp	Gly	Arg	Tyr	Ala	Leu	Ile	Phe
								420							
GGA	TCC	TTG	ATC	GCT	TCC	TAC	TAC	GCG	CAG	CTC	TTT	GTG	CCT	TTC	GTT
Gly	Ser	Leu	Ile	Ala	Ser	Tyr	Tyr	Ala	Gln	Leu	Phe	Val	Pro	Phe	Val
								480							
GTC	GAA	CGC	ACA	TGG	CTT	CAG	GTG	GTG	TTT	GCA	ATC	ATC	ATG	GGA	TTT
Val	Glu	Arg	Thr	Trp	Leu	Gln	Val	Val	Phe	Ala	Ile	Ile	Met	Gly	Phe
								540							
GCG	TGC	GCA	CAA	GTC	GGA	CTC	AAC	CCT	CTT	CAT	GAT	GCG	TCT	CAC	TTT
Ala	Cys	Ala	Gln	Val	Gly	Leu	Asn	Pro	Leu	His	Asp	Ala	Ser	His	Phe
								600							
TCA	GTG	ACC	CAC	AAC	CCC	ACT	GTC	TGG	AAG	ATT	CTG	GGA	GCC	ACG	CAC
Ser	Val	Thr	His	Asn	Pro	Thr	Val	Trp	Lys	Ile	Leu	Gly	Ala	Thr	His
								660							
GAC	TTT	TTC	AAC	GGA	GCA	TCG	TAC	CTG	GTG	TGG	ATG	TAC	CAA	CAT	ATG
Asp	Phe	Phe	Asn	Gly	Ala	Ser	Tyr	Leu	Val	Trp	Met	Tyr	Gln	His	Met
								720							
CTC	GGC	CAT	CAC	CCC	TAC	ACC	AAC	ATT	GCT	GGA	GCA	GAT	CCC	GAC	GTG
Leu	Gly	His	His	Pro	Tyr	Thr	Asn	Ile	Ala	Gly	Ala	Asp	Pro	Asp	Val

FIG. 7B

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TCG Ser	ACG Thr	TCT Ser	GAG Glu	CCC Pro	GAT Asp	GTT Val	CGT Arg	CTC Arg	ATC Ile	AAG Lys	CCC Pro	AAC Asn	CAA Gln	AAG Lys	TGG Trp
780	*														
TTT Phe	GTC Val	AAC Asn	CAC His	ATC Ile	AAC Asn	CAG Gln	CAC His	ATG Met	TTT Phe	GTT Val	CCT Pro	TTC Phe	CTG Leu	TAC Tyr	GGA Gly
CTG Leu	CTG Leu	GCG Ala	TTC Phe	AAG Lys	GTG Val	CGC Arg	ATT Ile	CAG Gln	GAC Asp	ATC Ile	AAC Asn	ATT Ile	TTG Leu	TAC Tyr	TTT Phe
GTC Val	AAG Lys	ACC Thr	AAT Asn	GAC Asp	GCT Ala	ATT Ile	CGT Arg	GTC Val	AAT Asn	CCC Pro	ATC Ile	TCG Ser	ACA Thr	TGG Trp	CAC His
ACT Thr	GTG Val	ATG Met	TTC Phe	TGG Trp	GGC Gly	GGC Gly	AAG Lys	GCT Ala	TTC Phe	TTT Phe	GTC Val	TGG Trp	TAT Tyr	CGC Arg	CTG Leu
ATT Ile	GTT Val	CCC Pro	CTG Leu	CAG Gln	TAT Tyr	CTG Leu	CCC Pro	CTG Leu	GGC Gly	AAG Lys	GTG Val	CTG Leu	CTC Leu	TTG Leu	TTC Phe
1020	*														
ACG Thr	GTC Val	GCG Ala	GAC Asp	ATG Met	GTG Val	TCG Ser	TCT Ser	TAC Tyr	TGG Trp	CTG Leu	GCG Ala	CTG Leu	ACC Thr	TTC Phe	CAG Gln

FIG. 7C

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1080
 GCG AAC CAC GTT GTT GAG GAA GTT CAG TGG CCG TTG CCT GAC GAG AAC
 Ala Asn His Val Val Glu Glu Val Gln Trp Pro Leu Pro Asp Glu Asn
 1140
 GGG ATC ATC CAA AAG GAC TGG GCA GCT ATG CAG GTC GAG ACT ACG CAG
 Gly Ile Ile Gln Lys Asp Asp Trp Ala Ala Met Gln Val Glu Thr Thr Gln
 1200
 GAT TAC GCA CAC GAT TCG CAC CTC TGG ACC AGC ATC ACT GGC AGC TTG
 Asp Tyr Ala His Asp Ser Ser His Leu Trp Thr Ser Ile Thr Gly Ser Leu
 AAC TAC CAG GCT GTG CAC CAT CTG TTC CCC AAC GTG TCG CAG CAC CAT
 Asn Tyr Gln Ala Val His His Leu Phe Pro Asn Val Ser Gln His His
 1260
 TAT CCC GAT ATT CTG GCC ATC ATC AAG AAC ACC TGC AGC GAG TAC AAG
 Tyr Pro Asp Ile Leu Leu Ile Ile Lys Asn Thr Cys Ser Glu Tyr Lys
 1320
 GTT CCA TAC CTT GTC AAG GAT ACG TTT TGG CAA GCA TTT GCT TCA CAT
 Val Pro Tyr Leu Val Val Lys Asp Thr Phe Trp Gln Ala Phe Ala Ser His
 1380
 TTG GAG CAC TTG CGT GTT CTT GGA CTC CGT CCC AAG GAA GAG TAGA
 Leu Glu his Leu Arg Val Leu Gly Leu Arg Pro Lys Glu Glu
 1440
 AGAAAAAAG CGCCGAATGA AGTATTGCC CTTTTTCTC CAAGAATGGC AAAAGGAGAT
 CAAGTGGACA TTCTCTATGA AGA

FIG. 7D

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MA29	MGIDOGI-KTFTW	10	20	30	40	50	60	70	59
MA524	MAAAPSVRTFTRAEVLNAEALNEGKKDAEAP--FLMIIDNKVYDVREFVDPDHPGGSVILTHV-GKDGTDV								67
BorD6	MA-----AQIKKYITSDDELKNHDKPGDLWISIQGKAYDVSDWVKDHPGGSFPLKSLAGQEVTDA								59
Sy6803D6	ML-TAE-RI-----								7
Sp1D6	MTSTTS-KV-----								8

MA29	FEMYHAFGAADAIMKKYYVGTLSNELPIFPEPTVFHKTIKTRVEGYFTDRNIDPKNRPEIWGRYALIFG	80	90	100	110	120	130	140	129
MA524	FDTFHIP-EAAWETLANFYVGDIDESDRDI--KNDDFAAEV-RKLRTLQSLGYDSSKAYYAFKVSFNLC								133
BorD6	FVAFHIP-ASTWKNLDKFFTGYYL--KDY--SVSEVSKDY-RKLVFEFSKMGLYDKK--GHIMFATLC								118
Sy6803D6	-----KFTQKRGFRRVLNQRVDAVFAEHGLTQRDNPSMYLKTIIIVL								49
Sp1D6	-----TFGKSIGFRKELNRRVNAYLEAENISPRDNPPMYLKTIIILA								50

MA29	SLIASYYAQLFVPFVVERTWLQVFAIIMGFACAQVGLNPLHDASHFSVTHNPTVWKILGATHDFFNGAS	150	160	170	180	190	200	210	199
MA524	IWGL--STVIVAKWGQTSTLANVLSAALLGLFWQQCGW-LAHDFLHHQVFDQDRFWGDLFGALGGVCCQGF								200
BorD6	FIAMLFAMSVYGVLCFEGVLVHLFSGCLMGFLWIQSGW-IGHDAGHYMVVSDSRLNKFIMGIFAANCLSGI								187
Sy6803D6	WLFSAW--AFVLFAPVIFPVRLLGCMVLAIALAASFNVGHDANHNAYSSNPHINRVLGMYDFVGLSS								116
Sp1D6	WVVSAAW--TFVVFEGPDVLWMKLLGCIIVLGFGVSAVGFNI SHDGNHGGYSKYQWVNYLSGLTHDAIGVSS								117

FIG. 8A

	220	230	240	250	260	270	280											
MA29	YLVW	MYQ-HMLG	HHPY	TNIAGAD	PDVST	SEPD	VRR	IKPN	QKWF	VNH	INQH	MFV	---	PFLYG	256			
MA524	SSSW	WKDKHNT	-HHIA	APNVHGE	DPDID	THPL	LLTWSE	HALEM	FSDVP	DEEL	T-RMWS	RFMV	LNQT	WIFY	FP	267		
BorD6	SIGW	WKWNHN	-AHHI	ACNSLEY	DPDLQY	IPFL	VVSS	KFFG	SLTSH	FYEK	RLT	FDLS	SRFEV	SYQH	WTFYP	256		
Sy6803D6	FL	-WRYR	-HNYL	HHTYTNI	LGHD	VEIHG	---	D-GA	VRMS	PE	QEHV	GIVY	RFQ	QFYI	---	WG	LYL	170
Sp1D6	YL	-WKFR	-HNVL	HHTYTNI	LGHD	VEIHG	---	D-EL	VRMS	PS	MEYR	WYH	RYQ	HWFI	---	W	IFY	171

	290	300	310	320	330	340	350
MA29	L L A F	- - - K V R I Q D I N I L Y F V K T N D A I R V N P I S T W H T V M F W G G K A F F V W Y R L I V P L O Y	-	L P L G K V L L L F T V	322		
MA524	I L C F	A R L S W C L Q S I L F V L P N G Q A H K P S G A R V P - I S L V E Q L S L A M H W T W Y -	L A T M F L F I K D P V N M L V Y F L V	335			
BorD6	I M S A	A R L N M Y V Q S L I M L L T K - - - - - R N V S - Y R A Q E L L G C L V F S I W Y - - P L L V S C L P N W G E R I M F V I A	315				
Sy6803D6	F I P F	- - - Y W F L Y D V Y L V L N K G Y H D H K I P P F Q P L E L A S L L G I K L L W L G Y V F G L P L A L G F S I P E V L I G A S V	237				
Sp1D6	F I P Y	- - - Y W S I I A D V Q T M L F I K R Q Y H D H E I P S P T W V D I A T L L A F K A F G V A V F L I I P I A V G Y S P L E A V I G A S I	238				

	360	370	380	390	400	410	420	
MA29	ADMVSSYWLALTFQANHVV	EEVQWPLPDE	-NGIQKDWAAMQVET	TQDYAHDSHLWTS	ITGSLNYQAVHH		391	
MA524	SQAVCGNLLAIVFSLNHNGMP	VI - - - - SKEEAVDM	DFFTKQII	TGRD	VHPG-LFANWF	TGGLNYQIEHH	399	
BorD6	SLSVTG-MQVQVQFSLNHFS	SSVY - - - - V-GKP	KGNWFEKQTDG	TLDI	ISCP-PWMDWFHGG	LQFQIEHH	377	
Sy6803D6	TYMTYGI VVCTIFMLAHVL	ESTEF	LPDGESGA	IDDEWAI	CQIRTTANFATNNPF	WNWFCGGLNHQVTIHH	307	
Sp1D6	VYMTHGLVACVVVFMLAHV	IEPAEFLD	PDNL - - H	IJDDEWAI	AQVKTTVD	FFAPNNPIINWYVGG	LNYYQTVHH	306

FIG. 8B

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[illegible]

FIG. 8C

SCORES INIT1: 117 INITN: 225 OPT: 256
SMITH-WATERMAN SCORE: 408; 27.0% IDENTITY IN 441 aa OVERLAP

ma29gcg.pep
MGTDQGKT---FTWEELAAHNTKDDLLAIRGRVYDVTKFLSRHPGGVDTL¹⁰LLGAGRDVT⁵⁰

253538a
QGPTPRYFTWDEVAQRSGCEEERWLVIDRKVYNISEFTRRHPPGGSRVISHYAGQDAT¹⁰
30 40 50

ma29gcg.pep
253538a

ma29gcg.pep
253538a

	120	130	140	150	160	170
	RPEIWGRYALIFGSLIASYYAQLFVPFVERTWLQVVF-AIIMGFACAQVGLNPLHDASH					
	: : : : : : : : : : : : : : : : : : : : : :					
	ANHVF--FLLYLLHILLDGAAWLTLVWFGTSLPFLCAVLLSAVQAQAGWLQ-HDYGH					

[illegible]

FIG. 9A


```

ma29gcg.pep      230          240          250          260          270          280
- - - PDVRR IKPNQKWF - VNHI NQHMFV - - PFLYGLL AFKVR IQDIN ILYFVKTNDAIRV
      : | : | : | : | : | : | : | : | : | : | : | : | : | : | : | :
253538a         LGWQP IEYGKKKLKYL PYNHQHEYFFL IGPPLL IPMYFYQYQI - - - IMTMI VHKNWVDL
230              240          250          260          270          280

```

ma29gcg.pep
NP ISTWHTVMFWGGKAFFVWYRLIVPLQLYPLGKVL³¹⁰LLFTVADMVSSYWLALTFQANHVV³⁴⁰
253538a
- - - - -AWAVSYYI - - - - -RFFITY - - - - -IPF-YGILG-ALLFLNFI³¹⁰RFL³²⁰ESHWFWVTQMNHIV³³⁰

ma29gcg.pep
EEVQWPLPDENG I QKDWAAMQVETT - - - QDYAHDSHLWTS ITGSLNYQA VHHLPNV S
| : | : : | : | : | : | : | : | : | : | : | : | : | : | : | : | : | : | :
MEI - - - - DQEAY - - RDWFSSQLTATCNVEQSFFND - - - WFS - - GHLNFQIEHHLFPTMP

[illegible]

FIG. 9B

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SCORES INIT1: 231 INITN: 499 OPT: 401
SMITH-WATERMAN SCORE: 620; 27.3% IDENTITY IN 455 aa OVERLAP

ma524gcg.pep	MAAAPSVRTFTRAEVLNAEALNEGKKDAEAPFLMI	DNKVVYDVREFVDPDHPGGSVILTH-
	:	: : : :
253538a	QGPTPRYFTWDEV - - - - - AQRSGCEERWLVI	DRKVYNI SEFTRRHPPGGSRVISHY
	10 20 30 40 50	
ma524gcg.pep	VGKDGTDVFDTFHPEAAW - - ETLANFYVGDI	DE - - - SDRDIKNDDFAAEVRKLRTLFQSL
	: : : : : : : : :	: : : : : : : : :
253538a	AGQDATDPFVAFHINKGLVKKYMNLSLLIGELS	PEQPSFEPTKNKELTDEFREL RATVERM
	60 70 80 90 100 110	
ma524gcg.pep	GYDSSKAYYAFKVSFNLCIWGLSTVI	VAKWGQSTLANVLSAALLGLFWQQCGWL AHDF
	: : : : : : : : : : :	: : : : : : : : :
253538a	GLMKANHVFLLYLLHILLDGAAWLTLWVFG -	TSFLPFLLCVLLSAVQAQAGWLQHDY
	120 130 140 150 160	
ma524gcg.pep	LHHQVFQDRFWGDLFGAF	LGGVCQGFSSWWKDKHNTHHAAPNVHG
	: : : : : : : : :	: : : : : : : : :
253538a	GHLSVYRKPKWNHLVHKFV	IGHLKGASANWNHRRHFQHHAKPNI
	170 180 190 200 210 220 230	FHKDPDVN - - - ML - - -

FIG. 10A

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SCORES INIT1: 231 INITN: 499 OPT: 401
SMITH-WATERMAN SCORE: 620; 27.3% IDENTITY IN 455 aa OVERLAP

ma524gcg.pep 240 250 260 270 280 290
EHALEMFS DVPDEELTRMWSRFMVNLQTWYFPILS - - - FARLSWCLQSILFVLPNGQAH
| : : : : | : : : : | : : : : | : : : : | : : : : | : : : : |
253538a -HVF-VLGEWQP I EYGKKKLLKYL PYNHQHEYFFLIGPPLLIPMYFQYQIMTMI - - - - VH
230 240 250 260 270

ma524gcg.pep 300 310 320 330 340 349
KPSGARVP I SLVEQLSLAMHWTWYLATMFLFIK - - DPVNMLVYFLVSQAVCGNLLAIVFS
| : : : : | : : : : | : : : : | : : : : | : : : : | : : : : |
253538a K - - - - - - - - - - NWVDLAWAVSYIRFFITYIPFYGILGALLFLNFI RFLESHWFVWVTQ
280 290 300 310 320

ma524gcg.pep 350 360 370 380 390 400 409
LNHNGMPVISKEEAVDMDFFTKQIITGRDVHPGLFANWFTGGLNYQIEHHLFSPMPRHNF
: | | : : : : | : : : : | : : : : | : : : : | : : : : | : : : : | : : : : |
253538a MNHIVMEI - - DQEAYR - DWFSSQLTATCNVEQSFFNDWFSGHLNFQIEHHLFPTMPRHNL
330 340 350 360 370 380

ma524gcg.pep 410 420 430 440 450
SKIQPAVETLCKKYNVRYHTTGMIEGTAEVFSRLNEVSKAASKMGKAQX
| | : : : : | | : : : : | : : : : | : : : : | : : : : |
253538a HKIAPLVKSLCAKHGIEYQEKPLLRALDII RSLKSGKLWLDAYLHKX
390 400 410 420 430

FIG. 10B

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/07421

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/53 C12N15/82 C12N5/10 C12P7/64 C11B1/00
A61K31/20 A23L1/30 A23K1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12P C11B A61K A23L A23K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 06712 A (RHONE POULENC AGROCHIMIE) 15 April 1993 cited in the application see the whole document ---	20-22
X	WO 94 18337 A (MONSANTO CO ;UNIV MICHIGAN (US); GIBSON SUSAN IRMA (US); KISHORE G) 18 August 1994 * see the whole document, esp. claims 8-10 *	20-47
X	WO 96 21022 A (RHONE POULENC AGROCHIMIE) 11 July 1996 cited in the application * see the whole document, esp. p. 2 1.3-21 * --- -/--	20-47

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search

21 August 1998

Date of mailing of the international search report

03/09/1998

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Authorized officer

Kania, T

INTERNATIONAL SEARCH REPORT

In Application No

PCT/US 98/07421

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 561 569 A (LUBRIZOL CORP) 22 September 1993 cited in the application see the whole document ---	20-47
A	COVELLO P. ET AL.: "Functional expression of the extraplastidial Arabidopsis thaliana oleate desaturase gene (FAD2) in Saccharomyces cerevisiae" PLANT PHYSIOLOGY, vol. 111, no. 1, May 1996, pages 223-226, XP002075211 see the whole document ---	1-51
A	WO 94 11516 A (DU PONT ;LIGHTNER JONATHAN EDWARD (US); OKULEY JOHN JOSEPH (US)) 26 May 1994 cited in the application see the whole document ---	1-51
T	WO 97 30582 A (CARNEGIE INST OF WASHINGTON ;MONSANTO COMPANY INC (US); BROUN PIER) 28 August 1997 see the whole document -----	1-51

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/07421

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 23, 42, 43
are directed to a method of treatment of the human/animal
body, the search has been carried out and based on the alleged
effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/ US 98 /07421

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (group of) inventions in this international application, as follows:

1. Claims 1-47, 49,50

Nucleic acid constructs comprising delta-5, delta-6, or delta-12 desaturases according to SEQ ID NO: 1,3,5, derived from the fungus *Mortierella alpina*.

Recombinant plant cells comprising said constructs.

Methods for obtaining altered long chain polyunsaturated fatty acid biosynthesis using plants comprising delta-5, delta-6, or delta-12 desaturases, or combinations thereof, derived from fungi or algae.

Plant oils derived from said plants and their use for therapeutical, nutritional, and cosmetical purposes, as well as products derived therefrom.

2. Claim : 48

An isolated sequence comprising the nucleotide sequence selected from the group of SEQ ID NO: 38-44, wherein said nucleotide is expressed in a plant cells.

3. Claim : 51

An isolated nucleotide sequence selected from the group consisting of SEQ ID NO: 49-50, wherein said sequence is expressed in a plant cell.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/07421

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		JP 8503364 T	16-04-1996

INTERNATIONAL SEARCH REPORT

Information on patent family members

Ir Application No

PCT/US 98/07421

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
W0 9730582 A	28-08-1997	AU 2050497 A	10-09-1997

